INTERCELLULAR ADHESION MOLECULE-1 GUIDES NAÏVE T CELL DIFFERENTIATION AND REGULATORY T CELL INDUCTION

By

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Submitted to the graduate degree program in the Department of Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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INTERCELLUAR ADHESION MOLECULE-1 GUIDES NAÏVE T CELL DIFFERENTIATION AND REGULATORY T CELL INDUCTION

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Chairperson Stephen H. Benedict, Ph.D.

Date approved: December 10, 2012

ABSTRACT

The outcome of naïve T cell activation and differentiation is guided by cues received from the cellular microenvironment, including cytokine and costimulatory signals. Naïve T cell precursors can differentiate into a variety of cellular subsets, each with a unique function in the immune response. In this Dissertation, we provide supporting evidence for the participation of costimulatory molecules in cell fate decisions. Using an *in vitro* differentiation system, we demonstrate that costimulation through Intercellular Adhesion Molecule-1 (ICAM-1) on the naïve CD4+ T cell can promote differentiation to Foxp3^{hi}CD25+CD127^{lo} regulatory T (T_{reg}) cells with suppressor function. These results add to the evidence previously published by our lab that ICAM-1 can act as a costimulatory molecule in the process of activation and differentiation of naïve CD4+ T cells from younger individuals. However, when naïve CD4+ T cells from older individuals were costimulated through ICAM-1, differentiation to effector and memory subsets was promoted, while differentiation to a T_{reg} subset was impaired. These data contribute to the concept that aging may alter naïve T cell activation and differentiation. Next, we examined the *in vivo* role of ICAM-1 using mice deficient in ICAM-1 or doubly deficient in ICAM-1 and CD28. ICAM-1$^{-/-}$ mice tended to have slightly increased CD8+ T cell function compared to wild-type controls, while ICAM-1$^{-/-}$CD28$^{-/-}$ mice displayed decreased CD8+ T cell function during acute viral infection. However, a lack of both ICAM-1 and CD28 did not completely abolish CD8+ T cell IFN-γ production or cytotoxicity. Finally, we further compared costimulation of human naïve CD4+ T cells through ICAM-1 and CD28 and identified potential differences in the timing of activation, in cytokine secretion, and in kinase signaling. Together, our results suggest that costimulation through ICAM-1 can participate in the differentiation of

T_{reg} cells from naïve precursors, and that differences in the costimulatory signal can influence differentiation outcome.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

Overview of the Immune System

The immune system is a complex group of cells and molecules whose primary purpose is to protect us from disease. Leukocytes are formed in the bone marrow during hematopoiesis, with multi-potent progenitor cells eventually giving rise to T cells, B cells, NK cells, dendritic cells, macrophages, neutrophils, eosinophils, and basophils (1). Each cell type has a unique and essential role in the immune response. While the innate immune system provides an immediate, non-specific defense against immune challenges, the adaptive immune system provides a response that is specific, diverse, can distinguish self from non-self, and can create immunological memory. The adaptive immune response is controlled by the actions of helper T (T_H) cells that secrete cytokines to influence the activation, differentiation, and function of cytotoxic T (T_C) cells, other T_H cells, B cells, and macrophages (2). Immune responses are kept in balance by CD4+ Regulatory (T_{reg}) cells that suppress the actions of other leukocytes (3).

T Cell Subsets

Cells destined to become T cells travel from the bone marrow to the thymus to undergo selection and maturation. Naïve T cells emigrate from the thymus and travel between the blood and peripheral lymphoid organs in search of cognate antigen. Upon antigen encounter, they differentiate into effector T cells. It was thought that memory T cells differentiated directly from effector T cells using a linear differentiation pathway, but more recent evidence suggests that memory T cells might arise from naïve T cells through a divergent differentiation pathway (4). T cells can also be divided into subsets of CD4+ T_H cells or CD8+ T_C cells. CD4+ T cells are classified by the cytokines that they produce and lineage-specific transcription factors they express. T_{H1} cells produce cytokines such as IFN-γ and IL-12 that promote cell-mediated

immune responses, T_{H2} cells produce cytokines such as IL-4 and IL-5 that promote humoral immune responses, and T_{H17} cells produce IL-17 which promotes an inflammatory response (2). Another type of CD4+ T cells, the T_{reg} cell has an essential role in immune tolerance. Natural T_{reg} cells arise in the thymus, while inducible/adaptive T_{reg} cells are induced in the periphery from naïve T cells activated under tolerogenic conditions (5).

T Cell Activation and Differentiation

Naïve T cells that receive a TCR signal plus a costimulatory signal become activated, proliferate, and differentiate into cell types that can participate in the immune response. TCR engagement occurs with MHC Class I (for CD8 T cells) or II molecules (for CD4 molecules) on APCs presenting cognate antigen, along with costimulatory molecule ligation. This interaction is known as the immunological synapse and involves segregation of receptors into supramolecular activation complexes (*i.e.* cSMAC, pSMAC, and dSMAC) (6). Signal 1 initiates intracellular signaling cascades that begin with activation of the Src kinase Lck, which phosphorylates Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on the CD3 and ζ chains associated with the TCR (7). Progressive phosphorylation steps lead to activation of Ras and PKC and release of intracellular calcium stores. Further downstream signaling leads to activation of AP-1, NF-κB, and NFAT. These transcription factors translocate to the nucleus and activate transcription of genes important in T cell activation, such as IL-2 (8).

Differentiation of naïve CD4+ T cells to effector cytokine-secreting cells, memory cells, and inducible regulatory T (T_{reg}) cells is described in **Figure 1.1** (4). Differentiation of naïve CD8+ T cells to effector cytotoxic T lymphocytes (CTLs) and memory cells during viral infection is described in **Figure 1.2** (9).

Costimulatory Molecules

Positive costimulatory molecules include CD28, CD2, CD5, CD9, CD27, CD44, CD46, CD81, LFA-1, VLA-4, OX40, 4-1BB, CD40L, LIGHT, SLAM, ICOS, and ICAM-1 (10, 11). According to the two-signal hypothesis, T cell activation, proliferation, and differentiation will occur if a naïve T cells receives Signal 1 from the TCR plus Signal 2 from a costimulatory molecule. However, if a naïve T cell receives Signal 1 without Signal 2, the T cell will become anergic or apoptotic (12, 13).

CD28 is the best characterized costimulatory molecule. CD28 is an Immunoglobulin (Ig) Superfamily member, containing one extracellular Ig domain, and expressed as a homodimer on T cells (13). The ligands of CD28 are B7.1 (CD80) and B7.2 (CD86). These ligands can also bind to the negative costimulatory molecule CTLA-4, which provides another method of regulating T cell activation. The intracellular tail of CD28 has been shown to interact with PI3K, Vav1, ITK, TEC, Grb-2, Lck, and PKCθ to amplify TCR signaling (13). However, ligation of CD28 alone without TCR triggering can activate components of the PI3K pathway (14).

Leukocyte Function-associated Antigen-1 (LFA-1) is a member of the β2 Integrin Subfamily consisting of a heterodimer of CD11a and CD18 molecules. LFA-1 is expressed by a variety of leukocytes, and has functions in cellular adhesion, leukocyte extravasation from the bloodstream, and as a costimulatory molecule. LFA-1 exists in an inactive conformation on resting leukocytes, but can undergo conformational change to an active form in the process of "inside-out" signaling. The primary ligands of LFA-1 are ICAM-1, -2, and -3, and JAM-1 (15).

Intercellular Adhesion Molecule-1 (ICAM-1) is a member of the Immunoglobulin Superfamily (IgSF), containing 5 extracellular Ig domains, a transmembrane domain, and a short cytoplasmic domain (16). ICAM-1 may also be expressed as alternatively spliced isoforms (17).

ICAM-1 is expressed on a variety of immune and non-immune cell types including leukocytes, endothelial cells, epithelial cells, fibroblasts, and keratinocytes . ICAM-1 expression can be upregulated after cellular activation since the ICAM-1 promoter contains binding sites for transcription factors such as AP-1, NF-κB, and STAT (18). The primary ligands of ICAM-1 are the β2 integrins LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18) and gp150/95 (CD11c/CD18). LFA-1 binds to the ICAM-1 amino-terminal domain (domain 1) (19), Mac-1 binds to domain 3 (20), and p150,95 binds to domain 4 (21). Rhinoviruses and erythrocytes infected with the malaria parasite *Plasmodium falciparum* use ICAM-1 as a cellular receptor, with binding occurring at distinct sites on domain 1 (22, 23).

Figure 1.3 shows the sequence alignment of the human and mouse ICAM-1 cytoplasmic domains. The human ICAM-1 cytoplasmic domain contains an RKIKK α-actinin binding sequence (24), an IKKYLRQ SHP-2 binding sequence (25), a putative SH3 domain-interacting PxxP motif (26), and putative phosphorylation sites (24). Previous data from our lab suggest the association of signaling proteins such as Lck and Erk1/2 with an ICAM-1 signaling complex (27). Our lab also previously found that stimulation of ICAM-1 alone on the Molt-3 T cell line could result in the transient inactivation of cdc2 kinase (28). ICAM-1 signaling in APCs has been shown to involve the RhoA family of G-proteins, Abl tyrosine kinase, and Src-family kinases (29). ICAM-1 expression on the vascular endothelium is important for leukocyte extravasation, and ICAM-1 expressed on the surface of APCs has been shown to be important as a costimulatory ligand for LFA-1 on the T cell. Our lab previously published that ICAM-1 expressed on the T cell surface itself could function as a costimulatory molecule (30).

Aging and the Immune System

The aging process causes alterations to cells of the immune system and can impair responses to challenges such as infections and tumors. Thymus involution that occurs through life leads to decreases in naïve T cell development (31). Additionally, there are defects in activation of the naïve T cells that are present in older individuals (32). Some biomarkers of immunosenescence that predict morbidity and mortality have been identified, known as immune risk profiles/phenotypes (IRPs). These include a CD4:CD8 ratio <1, the presence of CD28(-) T cells, decreased T cell proliferation, decreased B cell levels, and chronic infection with cytomegalovirus (32, 33) . Some proposed therapies to increase T cell responsiveness to antigen are to increase expression of costimulatory molecules on APCs or tumors, add specific cytokines, or use adjuvants that stimulate TLRs.

Regulatory T Cells

T cell tolerance to self-antigens occurs through several mechanisms including thymic negative selection, T cell anergy, T cell ignorance, and T_{reg} cell function (3). Although there may be several subsets of T_{reg} cells, the two most studied T_{reg} subsets are natural T_{reg} cells that develop in the thymus and inducible/adaptive T_{reg} cells that differentiate in the periphery. These two subsets share many phenotypic markers, but natural T_{reg} cells may be distinguished by expression of the transcription factor Helios (34). T_{reg} cells suppress the activation and function of other leukocytes by contact-mediated mechanisms (*e.g.* perforin, granzyme B, and surface-bound TGF-β (35-37)) and secretion of IL-10 (38).

Foxp3 is a transcription factor found primarily in T_{reg} cells. It functions as a transcriptional repressor, for example, inhibiting IL-2 transcription. Patients with *FOXP3* mutations develop a lethal autoimmune condition known as IPEX (Immune Deregulation, Polyendocrinopathy, Enteropathy, X-linked syndrome) (39). Foxp3-mutant scurfy mice and

Foxp3-null mice also develop a lethal lymphoproliferative autoimmune syndrome due to a lack of T_{reg} cell development (40).

Some of the signaling pathways involved in Foxp3 upregulation are the Smad pathway after TGF-β signaling, the Stat5 pathway after IL-2 binding, activation of AP1, NFAT, and CREB/ATF after TCR signaling, and inhibition of the Akt/mTOR pathway due to low levels of costimulation (41). Foxp3 expression is stabilized by epigenetic mechanisms such as histone acetylation and DNA methylation of the *FOXP3* locus (41). Important domains of Foxp3 protein include a proline-rich region with repressor function, a C2H2 zinc finger domain, a leucine zipper domain that allows for homodimerization, and a winged-helix/forkhead (FKH) domain that allows for nuclear translocation and transcriptional repression (42, 43).

Some clinical trials have begun to test the safety of adoptive T_{reg} therapy in preventing acute graft versus host disease (GVHD) (44). In addition, results from animal models of autoimmune diseases suggest that adoptive T_{reg} therapy might also be beneficial in the treatment of autoimmune diseases and asthma (45). Clinical trials have also been conducted to determine if T_{reg} depletion might allow for better anti-tumor responses during cancer treatment (46).

Figure 1.1. Summary of naïve CD4+ T cell activation and differentiation. A naïve CD4+ T cell encounters an APC expressing cognate antigen expressed in the context of MHC Class II. The naïve CD4+ T cell undergoes clonal expansion and differentiation to populations of effector CD4+ T_H cells or T_{reg} cells. These effector CD4+ T cells will eventually die by apoptosis. However, a subset of memory CD4+ T cells will remain that can function during a second encounter with the same antigen.

Figure 1.1

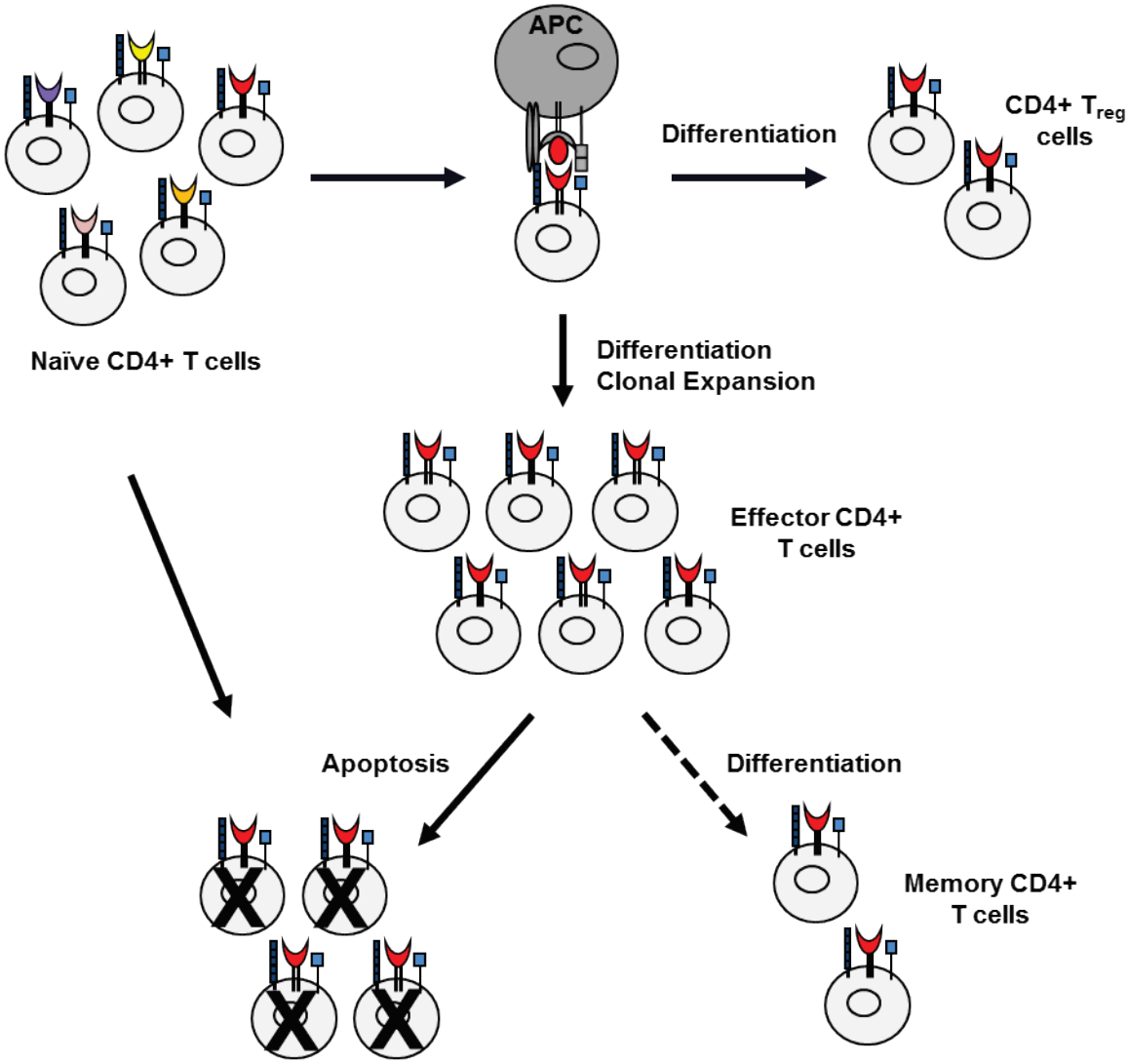

APC
Differentiation
CD4+ T_{reg} cells
Naïve CD4+ T cells
Differentiation
Clonal Expansion
Effector CD4+
T cells
Apoptosis
Differentiation
Memory CD4+
T cells

Figure 1.2. Summary of naïve CD8+ T cell activation and differentiation in response to viral infection. A naïve CD8+ T cell encounters an APC or virus infected cell expressing cognate antigen expressed in the context of MHC Class I. The naïve CD8+ T cell undergoes clonal expansion and differentiation to a population of effector CTLs that will kill virus infected cells in the periphery. These effector CTLs will eventually die by apoptosis. However, a subset of memory CD8+ T cells will remain that can function during a second challenge with the same virus.

Figure 1.2

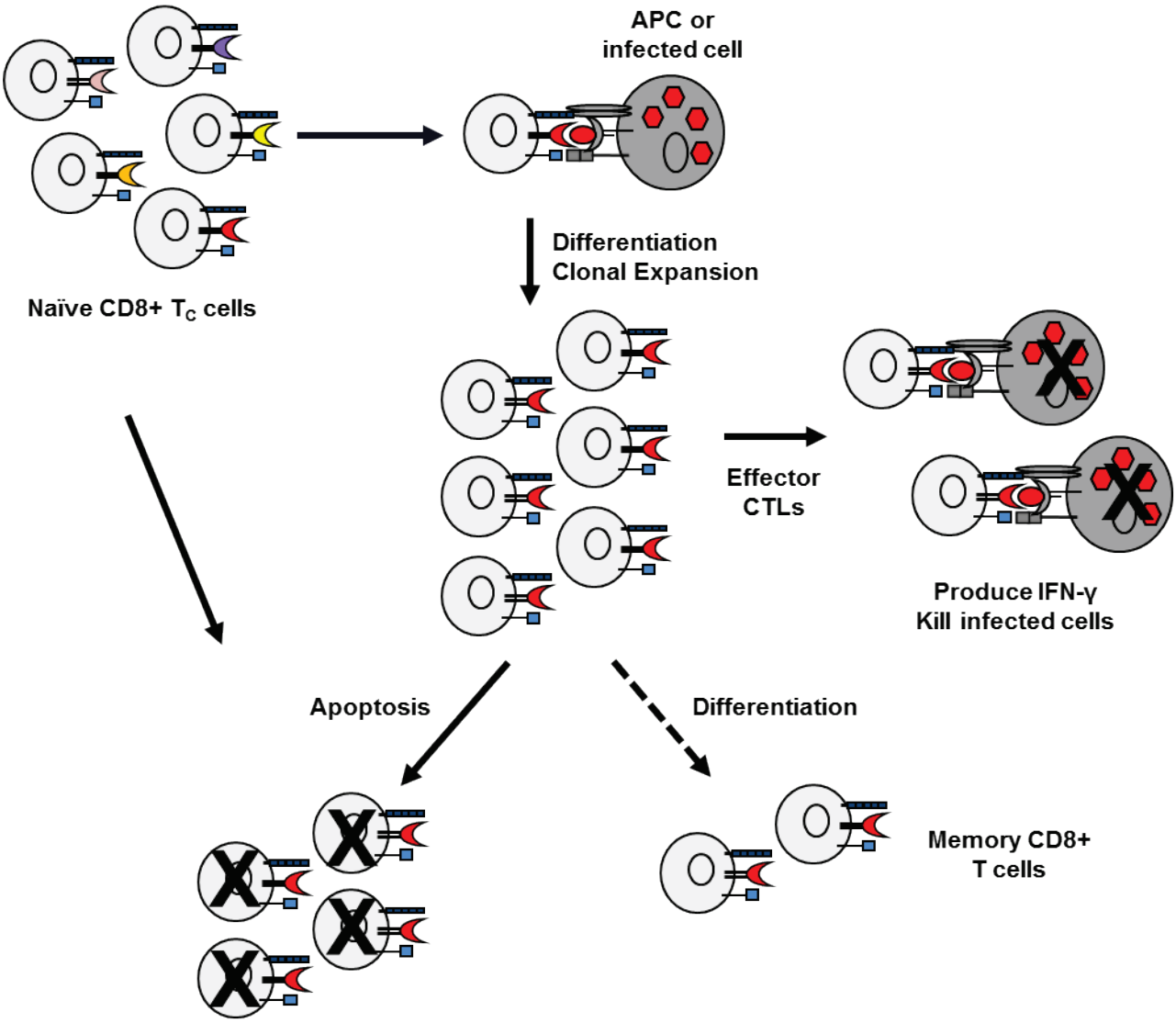
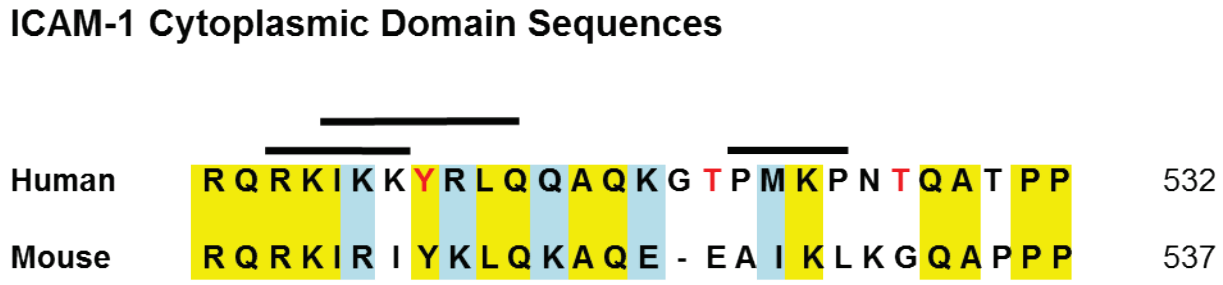

APC or
infected cell
Differentiation
Clonal Expansion
Naïve CD8+ T_C cells
Effector
CTLs
Produce IFN-γ
Kill infected cells
Apoptosis
Differentiation
Memory CD8+
T cells

Figure 1.3. Sequence alignment of human and mouse ICAM-1 cytoplasmic domains. Alignment was performed using BLAST Query ID: gi|825682|emb|CAA41977.1| (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Identical sequences are highlighted in yellow. Conservative substitutions (as determined by the BLAST substitution matrix) are highlighted in light blue. In the human sequence, putative phosphorylation sites are indicated in red (24), and the RKIKK α-actinin binding sequence (24), the IKKYLRQ SHP-2 binding sequence (25), and putative SH3 domain-interacting PxxP motif (26) are indicated with a bar above the corresponding sequences.

Figure 1.3


ICAM-1 Cytoplasmic Domain Sequences
Human
R Q R K I K K Y R L Q Q A Q K G T P M K P N T Q A T P P
532
Mouse
R Q R K I R I Y K L Q K A Q E - E A I K L K G Q A P P P
537

DISSERTATION OVERVIEW

ICAM-1 and Regulatory T Cell Induction

Our lab studies the ability of ICAM-1 expressed on the T cell surface to act as a costimulatory molecule to influence T cell activation and differentiation using an *in vitro* model of differentiation. This method uses plate-bound antibodies against CD3, which mimic a TCR signal, plus antibodies against ICAM-1 or against CD28. Former lab members have previously demonstrated differentiation of human naïve CD4+ T cells to effector and memory subsets after costimulation through ICAM-1 using this method (47). In this Dissertation, we report a new role for ICAM-1 in the induction of a subset of $Foxp3^{hi}CD25+CD127^{lo}$ T_{reg} cells with *in vitro* suppressor function (48).

Aging and T Cell Differentiation

We also assessed whether the process of aging might affect the potential for naïve CD4+ T cells to generate effector, memory, or T_{reg} cells. We compared costimulation of naïve CD4+ T cells from older individuals (age 65 years or older) with naïve CD4+ T cells obtained from younger individuals. While naïve CD4+ T cells from older individuals retained the ability to differentiate to effector and memory cells after ICAM-1 costimulation, they appeared to lose the ability to differentiate to T_{reg} cells.

ICAM-1 and Mouse T Cell Activation and Differentiation

To determine the role of ICAM-1 on T cell activation and differentiation *in vivo*, we studied whether ICAM-1 expressed on mouse T cells would function similarly to our results using human T cells. Attempting to stimulate mouse CD4+ T cells through CD3+ICAM-1 did not appear to lead to either increased proliferation or to T_{reg} induction. However, we observed

differences in CD8+ T cell function after viral infection of ICAM-$1^{-/-}$ mice, ICAM-$1^{-/-}$$CD28^{-/-}$ mice, and wild-type C57Bl/6 mice.

Costimulation and Differentiation Outcome

One of the overall goals of our lab is to determine how differential signaling through costimulatory molecules can lead to distinct cell fates. In this dissertation, we assessed the kinetics of activation, production of cytokines and chemokines, and expression and activation of kinases after costimulation through either ICAM-1 or CD28. Our results suggest that signaling and functional differences can result from costimulation through ICAM-1 compared to costimulation through CD28.

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CHAPTER 2

ICAM-1 CAN PARTICIPATE IN REGULATORY T CELL INDUCTION

ABSTRACT

Inducible regulatory T (T_{reg}) cells can be generated from naïve cells in the periphery after activation under tolerogenic conditions. The mechanisms of T_{reg} differentiation are still being characterized. We contributed to this characterization by investigating the participation of Intercellular Adhesion Molecule-1 (ICAM-1) in the process of T_{reg} induction beginning with human naïve CD4+ T cells. Costimulation of naïve CD4+ T cells through ICAM-1 *in vitro* without the addition of exogenous cytokines led to the formation of a population of Foxp3^{hi} CD25+ CTLA-4+ CD127^{lo} T_{reg} cells. In contrast, costimulation through CD28 under these conditions did not result in T_{reg} induction. The T_{reg} cells that differentiated following costimulation through ICAM-1 strongly inhibited responder cell proliferation in an *in vitro* suppression assay. We performed additional experiments to further describe the T_{reg} cells that formed after costimulation through ICAM-1. Our results 1) suggest that ICAM-1 resident on the surface of naïve CD4+ T cells plays a role in the differentiation of T_{reg} cells from naïve precursors, 2) support the hypothesis that signaling through different costimulatory molecules can lead to different functional outcomes, and 3) suggest that ICAM-1 might be a possible signaling molecule to target for T_{reg}-mediated therapies.

NOTES

Much of the work presented in this chapter was published by Williams *et al.* in 2011 in the journal Cellular Immunology (1).

A patent application covering some of the work presented in this chapter was submitted by The University of Kansas and was approved in 2012.

INTRODUCTION

The immune system employs several mechanisms to maintain peripheral tolerance including T cell anergy, T cell ignorance, and suppression by regulatory T (T_{reg}) cells (2). Several T_{reg} subsets have been identified and classified based on their site of development, mechanism of induction, or by the cytokines they produce (3, 4). One subset of T_{reg} cells develops in the thymus (natural T_{reg} cells) while a different subset differentiates from naïve precursors after leaving the thymus and encountering antigen in the periphery (inducible or adaptive T_{reg} cells). Natural T_{reg} cells are CD4+CD25+ and express the transcription factor Foxp3. The expression of Foxp3 among inducible T_{reg} subsets is variable. Inducible Tr1 cells are characteristically Foxp3(-), while T_{reg} cells induced in the presence of TGF-β or low-dose antigen are typically Foxp3+ (5). Although activation of T_{reg} cells occurs in an antigen-specific manner, they are able to suppress other leukocytes in an antigen-independent manner (6). This suppression is accomplished by both contact-mediated mechanisms involving perforin, granzymes, and surface-bound TGF-β (7-9) and through the immunosuppressive effects of soluble IL-10 independent of cell contact (10).

There is great interest in understanding the mechanisms by which T_{reg} cells are generated and in controlling the development and function of both natural and inducible T_{reg} cells for therapeutic purposes. Populations of mouse CD4+CD25(-) cells can differentiate into T_{reg} cells following *in vitro* stimulation with the combination of anti-CD3, anti-CD28 or antigen presenting cells, and TGF-β (11, 12), or by delivery of peptide *in vivo* under sub-immunogenic conditions (13, 14). Human T_{reg} cells have been induced using autologous cell stimulation in the presence of IL-4 or IL-13 (15), allogeneic cell stimulation in the presence of TGF-β (16, 17), stimulation with immature dendritic cells plus IL-2 (18), or stimulation with murine L fibroblast

cell transfectants expressing hCD32, hCD58, hCD80 plus anti-CD3 in the presence of IL-2, IL-15, IL-10 and IFNα (19).

It is likely that the differentiation of naïve T cells to T_{reg} cells is influenced by multiple factors including the cytokine environment and signaling through specific costimulatory molecules. TGF-β1 is a cytokine that polarizes naïve CD4+ T cells to the T_{reg} subset upon activation (11, 12, 16, 17). IL-2 has also been shown to participate in T_{reg} induction (20). Some costimulatory molecules have been implicated in the generation of T_{reg} cells. The inhibitory molecules CTLA-4 (21) and PD-1 (22), and the positive costimulatory molecules CD46 (23), CD52 (24), and CD2 (25) have been shown to promote T_{reg} induction. Although CD28 is necessary for natural T_{reg} development in the thymus, the requirement for CD28 for T_{reg} induction in the periphery has been controversial (26).

Our lab has previously shown that Intercellular Adhesion Molecule-1 (ICAM-1, CD54), resident on the T cell surface, can act as a classical costimulatory molecule to effect human naïve T cell activation and differentiation to effector and memory subsets (27, 28). In this chapter, we describe a function for ICAM-1 in T_{reg} induction. Our data show T_{reg} induction from human naïve CD4+ T cells after costimulation through ICAM-1, but not after costimulation through CD28. These data support the model that the specific costimulatory signal helps regulate the outcome of naïve T cell differentiation.

MATERIALS AND METHODS

Cell Purification

Human naïve CD4+ T cells were isolated from peripheral blood from healthy donors using Ficoll-Paque PLUS (GE Healthcare, Piscataway, NJ) density-gradient centrifugation followed by negative selection using a StemSep Human Naïve CD4+ T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC) as we have described previously (28). Refer to Protocol 1 in the Appendix for more detail. In selected experiments where indicated, naïve CD4+ T cells were isolated from human tonsil tissue. In summary, tonsils were minced over a strainer to obtain cell suspensions, mononuclear cells were purified using Ficoll-Paque PLUS density-gradient centrifugation, and total T cells were isolated from tonsil cell suspensions using E-rosetting using sheep RBCs as we have described previously (29). See Appendix Protocol 2 for a description of the procedure. Next, naïve CD4+ T cells were purified using negative selection with a StemSep Human Naïve CD4+ T Cell Enrichment Kit (StemCell Technologies). If the origin of the cells used in an experiment is not specifically given, it can be inferred that they were human naïve CD4+ T cells purified from peripheral blood. Naïve cells for this study were defined as CD4+CD45RA+CD45RO(-)CD11a^{lo}CD27+ and routinely were >98% CD45RA+ as determined by flow cytometry (**Fig. 2.1A**). In selected experiments where indicated, either CD25+ or CD25+CD127(-) T cells were purified from the stimulated cultures using either CD4+CD25+ Regulatory T Cell Isolation Kits or CD4+CD25+CD127(-) Regulatory T cell Isolation kits (Miltenyi Biotec, Auburn, CA).

Cell Culture Reagents

Cells were cultured in complete RPMI 1640 medium (Mediatech, Herndon, VA) containing heat-inactivated 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen/Life Technologies, Carlsbad, CA). No exogenous cytokines were added unless indicated.

Stimulating Antibodies

Anti-human ICAM-1 antibody clone R6.5D6 was either purified from serum-free hybridoma cultures (hybridoma purchased from ATCC, Manassas, VA) or purchased from BioXCell (West Lebanon, NH). Anti-CD3ε antibody clone OKT3 was either purified from serum-free hybridoma culture (hybridoma purchased from ATCC) or purchased from eBioscience (San Diego, CA). Anti-CD11a antibody clone HB202 was purified from serum-free hybridoma cultures (hybridoma purchased from ATCC). Anti-CD28 antibody clone ANC28.1 was purchased from Ancell (Bayport, MN) or clone CD28.2 was purchased from BD Biosciences (San Jose, CA). Clone ANC28.1 was used for most experiments. Antibodies were titrated to determine the optimal concentration. The concentration of anti-CD3 used was the highest concentration that did not induce proliferation of naïve CD4+ T cells unless the anti-CD3 antibody was combined with an anti-costimulatory molecule antibody. The concentration of the anti-costimulatory molecule antibodies were the lowest concentrations that gave the maximum proliferation when used with the selected anti-CD3 concentration. The following antibody concentrations were used: anti-CD3: 1 μg/mL, anti-ICAM-1: 10 μg/mL, anti-CD11a: 10 μg/mL, anti-CD28: 2-5 μg/mL (dependent on the lot of anti-CD28 antibody used). Antibodies were diluted in sterile PBS, added in 200 μL aliquots to flat-bottom 96-well plates, and then allowed to adhere for 2 hours at 37° C or overnight at 4° C. Plates were washed 3 times with sterile PBS

to remove unbound antibody. After washes, cell solutions were added to the 96-well flat-bottom plates at a concentration of $1.5x10^6$ cells/mL in 200 μL ($3x10^5$ cells per well). Cells were incubated in a 37 °C incubator containing a 5% CO_2 atmosphere for the indicated amounts of time. This procedure is also presented in Protocol 3 in the Appendix.

Flow Cytometry Antibodies

Anti-human Foxp3-PE used was either clone PCH101 from eBioscience or clone 3G3 from Miltenyi Biotec. In most experiments, clone PCH101 was used. Experiments were performed using Fixation/Permeabilization reagents and the accompanying protocol from eBioscience. Anti-CD25-FITC, anti-CD25-TriColor, anti-CD11a-FITC, anti-CD27-PE, CD62L-TriColor, anti-CD45RA-TriColor, anti-CD45RO-PE, anti-CD54 (ICAM-1)-PE, and anti-CD28-TriColor were from Caltag Laboratories/Life Technologies. Anti-CD127-PE, anti-CD152 (CTLA-4)-PE, anti-CCR7-PE, and anti-integrin β7-PE-Cy5 were from BD Biosciences. Anti-integrin α4-PE, and anti-integrin β1-PE-Cy5 were from eBioscience. Anti-LAP/TGF-β1-PE was from R&D Systems. Anti-phospho-p44/42MAPK-AlexaFluor488 was from Cell Signaling Technologies (Danvers, MA). Isotype control antibody for anti-Foxp3-FITC clone PCH101 was Rat IgG2a-FITC (eBioscience), isotype control antibody for anti-Foxp3-PE clone PCH101 was Rat IgG2a-PE (Caltag/Life Technologies), and isotype control antibody for anti-Foxp3-PE clone 3G3 was Mouse IgG1-PE (Caltag/Life Technologies). Isotype control antibody for anti-CD45RA-Tri was Mouse IgG2b-TriColor (Caltag/Life Technologies). Isotype control antibody for anti-phospho-p44/42MAPK-AlexaFluor488 was Mouse IgG1-AlexaFluor488.

Flow Cytometry Surface Staining

At the indicated times after costimulation in culture, cells were removed from the plate and centrifuged, supernates were aspirated, and cells were resuspended in 100 µL cold Staining Buffer (Dulbecco's PBS + 0.5% BSA). Cells were incubated 15 minutes on ice in the dark. Cells were centrifuged again, supernates were aspirated, and cells were resuspended in 100 µL cold Staining Buffer plus fluorescently-conjugated antibodies, and incubated on ice for 20 minutes in the dark. Cells were washed by adding 500 µL cold Staining Buffer, centrifuging, and aspirating supernatant. Cells were resuspended in 200-400 µL Staining Buffer plus 2-4% final percent paraformaldehyde to fix the cells. Cells were analyzed using either a BD FACScan or an Accuri C6 cytometer. Protocol 4 in the Appendix describes the surface staining procedure.

Intracellular Flow Cytometry Staining for Foxp3

Foxp3 flow cytometry staining was performed following the protocol outlined by the eBioscience tech sheet protocol, with modifications to the reagent volumes used as indicated here. Cells were first surface stained using the protocol described above, minus the final fixation step. Next, cells were placed in 400 µL eBioscience Fixation/Permeabilization Buffer and incubated for 30-60 minutes in the dark at 4° C in the dark. Cells were washed with 600 µL Permeabilization Buffer and resuspended in 100 µL Permeabilization Buffer plus 1 µL normal rat serum (corresponding to anti-Foxp3 clone PCH101) or normal mouse serum (corresponding to anti-Foxp3 clone 3G3) and incubated for 5 minutes on ice in the dark. Next, the titrated amount of anti-Foxp3 antibody was added and the tubes were gently vortexed to mix. After incubating for 30 minutes on ice in the dark, the cells were washed twice with 800 µL

Permeabilization Buffer. Cells were resuspended in 200-400 μL Staining Buffer plus 2-4% final percent paraformaldehyde.

Antibodies for Cytokine Blocking

Antibodies used for cytokine blocking experiments were anti-IL-10 (eBioscience), anti-IL-2, and anti-TGF-β1 (R&D Systems, Minneapolis, MN). Isotype control antibody for anti-IL-10 was Rat IgG1 and isotype control antibody for anti-IL-2 and anti-TGF-β1 was Mouse IgG1 (eBioscience).

Cytokine Removal by Protein G

Protein G Sepharose 4 Fast Flow (Amersham/GE) was centrifuged at 4° C, and the supernate aspirated. The Protein G pellet was resuspended at 50% in 100 mM Tris pH 8.0. This solution was centrifuged again, and the supernate aspirated. This wash was repeated 5 times. The Protein G pellet was resuspended at 50% in 100 mM Tris pH 8.0, and aliquoted into new Eppendorf tubes. The solution was centrifuged and supernates aspirated. The appropriate cell culture supernates were added to the tubes containing Protein G. The samples were gently agitated at 4°C for 60 minutes by shaking for 30 minutes and rotating for 30 minutes. The samples were centrifuged and the supernate was saved. Supernate cytokine concentrations were assayed using ELISA (IL-10 or TGF-β1 ELISA from R&D Systems). All steps were performed under sterile conditions. This protocol is included as Protocol 5 in the Appendix.

Addition of Exogenous Cytokines or Retinoic Acid

In selected experiments where indicated, the recombinant human cytokines TGF-β1 (R&D Systems) and IL-2 (Boehringer Mannheim) were added. TGF-β1 was used at 10 ng/mL and IL-2 was used at 10 U/mL. In selected experiments where indicated, retinoic acid was added. All-trans Retinoic Acid was purchased from Sigma-Aldrich (St. Louis, MO). It was dissolved in DMSO and used at a concentration range of 1 – 10 nM.

Additional Flow Cytometry Reagents

CFSE (5-(and-6)-carboxyfluoresceindiacetate, succinimidyl ester) was from Molecular Probes/Life Technologies (Carlsbad, CA) and used at 2.5 μM final concentration. Cells were labeled for 10 minutes at 37° C in the dark in serum-free RPMI1640 medium at a concentration of 2 million cells/mL. Cells were centrifuged at low speed for 5 minutes and supernatant aspirated. Cells were washed twice in complete RPMI1640 medium. This procedure is included in the Appendix as Protocol 6.

Flow Cytometry Analysis

Flow cytometry was performed using a FACScan (BD, San Jose, CA) or an Accuri C6 (Accuri Cytometers, Ann Arbor, MI). Data analysis was performed using CellQuest software (BD), CFlow (Accuri) and FlowJo software (Tree Star, Inc., Ashland, OR).

Cytokine ELISA

Cell culture supernates were collected from stimulated cultures and used after clarification by centrifugation. IL-10 production was measured using Human IL-10 ELISA Ready-Set-Go kits (eBioscience, San Diego, CA) or Human IL-10 Quantikine kits (R&D Systems, Minneapolis,

MN). Levels of secreted TGF-β1 were determined using Human TGF-β1 Quantikine kits (R&D Systems). The procedure for the Human TGF-β1 Quantikine ELISA first required activation of latent TGF-β1 to active TGF-β1 by addition of acid and then neutralization. Plates were analyzed using an Automated Microplate Reader (BioTek, Winooski, VT) and DeltaSoft software (BioMetallics Inc, Princeton, NJ).

Suppression Assay

Naïve CD4+ T cells were stimulated for 10 days using anti-CD3 plus anti-ICAM-1. On Day 10, the stimulated cells were spun over Ficoll-Paque (GE Healthcare, Piscataway, NJ) to remove dead cells. The CD4+CD25+ T_{reg} cells were separated from the CD4+CD25(-) cells using CD4+CD25+ Regulatory T Cell Isolation Kits (Miltenyi Biotec). Also on Day 10, fresh peripheral blood was again obtained from the same donor and second bleed total T cells were isolated using Ficoll-Paque density centrifugation and a Human T Cell Enrichment Kit (StemCell Technologies, Vancouver, BC). The cultured CD4+CD25+ T_{reg} cells, the cultured CD4+CD25(-) cells, and an aliquot of second bleed total T cells to be used as a control were each stained with PKH26 dye (Sigma, St. Louis, MO) at 2.5 μM concentration. An aliquot of second bleed total T cells to be used as responders was labeled with CFSE (Molecular Probes/Life Technologies) at 2.5 μM concentration. Subsequently, the cells were cultured at T_{reg} (or Control) Cell: Responder Cell ratios of 1:1, 1:2, and 1:4. Co-cultured cells were stimulated for 5 days using anti-CD3 plus anti-CD28 antibodies as described above ($3x10^5$ cells/well). Proliferation of the CFSE-labeled responder cell population was assessed using flow cytometry by gating out the PKH26-labeled T_{reg} or control populations and analyzing proliferation of the CFSE-labeled responder population.

Human Subjects

Peripheral blood cells were obtained after informed consent of healthy volunteers. Procedures were approved by The University of Kansas Institutional Review Board.

RESULTS

Costimulation of naïve CD4+ T cells through ICAM-1 induced cells with a T_{reg} phenotype

Our interest in differentiation outcome after costimulatory molecule ligation led us to investigate the possibility that costimulation through ICAM-1 might be involved in the induction of regulatory T cells. We employed our *in vitro* model of T cell activation and differentiation by costimulating human naïve CD4+ cells through the TCR/CD3 complex and either ICAM-1 or CD28 using plate-bound antibodies to mimic an antigenic signal plus a costimulatory signal. *In vitro* costimulation using plate-bound antibodies is a well-established method which allows us to focus on stimulating the cell-surface proteins of interest and avoid, as much as possible, additional signaling that could occur if antigen presenting cells provided the stimuli.

Naïve CD4+ T cells were obtained from human peripheral blood in most experiments, but in a few experiments, where indicated, naïve CD4+ T cells were purified from human tonsil tissue. Naïve CD4+ cells have been characterized phenotypically by other groups as CD45RA+CD45RO(-)CD11a^{lo}CD27+ (30) and also as expressing the molecules CD28, LFA-1, CCR7, CD62L, CD27, CD2, and VLA-4 (Very Late Antigen-4, integrin α4β1) (31). The naïve CD4+ T cell population we isolated using negative selection columns was CD45RA+CD45RO(-) and the gated CD45RA+ cells were CD11a^{lo}CD27+ (**Fig. 2.1A).** The naïve population also expressed the lymph node homing markers CCR7 and CD62L, the gut associated lymphoid tissue (GALT) homing marker LPAM-1 (Lymphocyte Peyer's Patch Adhesion Molecule-1, integrin α1β7) and VLA-4 (integrin α1β1) **(Fig. 2.1B)**. Expression of the costimulatory molecules of interest in our study, ICAM-1 and CD28, were expressed at a low but detectable level on the resting naïve CD4+ T cells **(Fig. 2.1C)**. Therefore, the phenotype of the starting naïve CD4+ T cells used in our studies corresponded with what has been previously reported in

the literature (30-32). The starting nonstimulated naïve CD4+ T cell population routinely contained less than 3% of Foxp3^{lo} cells (MFI = 29, using anti-Foxp3-PE clone PCH101, mean of 15 samples), and no Foxp3^{hi} cells (defined in stimulated samples as MFI = 236, using anti-Foxp3-PE clone PCH101, mean of 11 samples) **(Fig. 2.1D)**. The naïve cells expressed the IL-7Rα chain (CD127) but did not express the IL-2Rα chain (CD25) **(Fig. 2.1D)**, which is a naïve phenotype also previously reported (33).

To generate both an antigenic signal and a costimulatory signal, we stimulated the purified naïve CD4+ T cells in 96-well plates ($3x10^5$ cells/well) coated with immobilized antibodies directed against CD3 plus antibodies directed against different costimulatory molecules. The treatment groups usually were as follows: Nonstimulated, CD3 alone, CD3+ICAM-1, and CD3+CD28. No exogenous cytokines were added to the stimulated cultures.

Following our lab's investigation of the function of ICAM-1 in memory cell formation, we were interested in determining whether ICAM-1 was also involved in the differentiation of T_{reg} cells from naïve cells. Some inducible T_{reg} cells have been phenotypically characterized as CD4+ Foxp3+ CD25+ CTLA-4+ CD127^{lo} (5, 33-35). In addition, CD62L is expressed on some types of T_{reg} cells, and may help them home to and function in lymphoid organs (36, 37). CD62L is also expressed on other T cell subsets, including naïve T cells (**Fig. 2.1B**). CD25 is the IL-2Rα chain and CD127 is the CD127 is the IL-7Rα chain. The presence of CD25 and CTLA-4 and absence of CD127 is not a definitive phenotype for T_{reg} cells since this phenotype occurs on some activated CD4+ cells as well (38). The transcription factor Foxp3 is considered a more definitive T_{reg} marker since ectopic Foxp3 expression can convert CD4+CD25- cells to CD4+CD25+ regulatory cells (39-41). After approximately 5 days of stimulation using anti-CD3 plus anti-ICAM-1, we detected a subset of cells with a T_{reg} phenotype. This population

expressed high levels of the intracellular T_{reg} marker Foxp3 (**Fig. 2.2A**) and the cell-surface T_{reg} markers CD25 and CTLA-4, did not express CD127, and remained CD62L+ (**Fig. 2.2B**). In agreement with previous reports (42), we did not observe sizeable T_{reg} populations in the CD3 stimulated, or CD3+CD28 stimulated samples. Stimulation through ICAM-1 alone did not generate a T_{reg} population (data shown in **Fig. 2.3A**) suggesting that both an antigenic signal and a costimulatory signal are required for T_{reg} induction. In the few experiments when we stimulated using anti-CD3+anti-CD11a (LFA-1), we sometimes observed a small population of $Foxp3^{hi}$ cells after costimulation through CD3+CD11a , but did not observe this population in other experiments (data not shown). A large population of cells stimulated by any treatment appeared to express intermediate levels of Foxp3. This may indicate nonspecific Foxp3 antibody binding, or may correspond with an observation of other investigators in which activation promotes a slight transient increase in Foxp3 expression without full conversion to the T_{reg} subset (43, 44).

T_{reg} proliferation and kinetics of Foxp3 expression

Since we observed strong proliferation of naïve CD4+ T cells in response to ICAM-1 costimulation, we were interested in assessing proliferation of the T_{reg} population during the differentiation process. We stained newly purified naïve CD4+ T cells with CFSE and stimulated them using the indicated treatment regimens (**Fig. 2.3A**). After 7 days of stimulation, we observed that the $Foxp3^{hi}$ population included both undivided cells (upper right quadrant) and cells that had undergone cell division (upper left quadrant), suggesting that at least some of the differentiating T_{reg} cells had upregulated Foxp3 without dividing. For the cells that had both divided and upregulated Foxp3, we were unable to distinguish whether the cells differentiated and then divided, divided and then differentiated, or if both processes occurred concurrently.

Even though cells proliferated robustly with costimulation through CD28, we did not detect an appreciable population of Foxp3^{hi} cells with this stimulation condition. When we analyzed the kinetics of T_{reg} induction, we observed that the mean percentage of Foxp3^{hi} T_{reg} cells was highest at Day 7 of stimulation through CD3+ICAM-1 (**Fig. 2.3B**).

Role of cytokines in T_{reg} differentiation after ICAM-1 costimulation

Specific cytokines have been shown to have roles in both induction of T_{reg} cells and in the mechanisms by which T_{reg} cells suppress. IL-10 has been shown to induce some subsets of T_{reg} cells (19). Also, one mechanism of T_{reg} suppression is the production of the immunosuppressive cytokine IL-10 which functions to inhibit IL-2 production and proliferation of T cells (10, 45, 46). We collected cell culture supernates from naïve CD4+ T cells stimulated through CD3+ICAM-1 or through CD3+CD28. IL-10 ELISAs detected moderate amounts of IL-10 in supernates from cells costimulated through ICAM-1 (**Fig. 2.4A**). Interestingly, the mean concentration of IL-10 peaked on day 7, which corresponds to the day of the highest T_{reg} percentage in ICAM-1 costimulated cultures (**Fig. 2.3B**). TGF-β1 is another T_{reg}-associated cytokine that has been shown to be involved both in T_{reg} induction and in suppression by T_{reg} cells (9, 16, 42). Although TGF-β1 secretion was highly variable among 4 experiments conducted, both costimulation through ICAM-1 and through CD28 led to secretion of TGF-β1 **(Fig. 2.4B)**. Since we were detecting cytokine secretion by cells in the entire culture, we cannot determine whether the T_{reg} cells themselves were secreting either IL-10 or TGF-β1. We can only conclude that some cells in the cultures were secreting these cytokines after stimulation.

T_{reg} cells have been shown by other groups to use cell-surface TGF-β1 to suppress other leukocytes, and to express cell-surface TGF-β complexed with LAP (latency-associated peptide) (9, 47, 48). We detected expression of surface TGF-β1 using an antibody that would bind to

LAP. We found that some newly isolated naïve CD4+ T cells expressed LAP/TGF-β1, and that some of the Foxp3^{hi} cells generated after CD3+ICAM-1 stimulation expressed LAP/TGF-β1. However, some Foxp3^{lo} cells also expressed LAP/TGF-β1 after CD3+ICAM-1 stimulation and some cells expressed LAP/TGF-β1 after CD3+CD28 stimulation **(Fig. 2.4C)**. Therefore, in our experiments, LAP/TGF-β1 does not appear to be a T_{reg}-specific marker. We chose not to analyze secretion of IL-2 since a former graduate student in Dr. Benedict's lab, Dr. Jake Kohlmeier, had previously shown that costimulation through either CD3+ICAM-1 or CD3+CD28 led to production of high IL-2 levels (28).

Other investigators have shown that the addition of exogenous TGF-β1 plus IL-2 can upregulate Foxp3 expression due to TGF-β1 signaling through Smad3, and IL-2 signaling through STAT3 and STAT5 (20, 49, 50). In our stimulation regimen, we do not add exogenous IL-2 or TGF-β1, yet still observe a population of inducible T_{reg} cells. To determine if adding exogenous TGF-β1 plus IL-2 could further enhance T_{reg} induction in our cell culture system, we added 10 ng/mL TGF-β1 and 10 U/mL IL-2 to the culture medium. Addition of exogenous TGF-β1 plus IL-2 lead to an increase in the percentage of Foxp3^{hi} cells after costimulation through ICAM-1 (**Fig. 2.5**).

To determine if the T_{reg}-associated cytokines we detected after costimulation through ICAM-1 were necessary for the T_{reg} induction process, we used blocking antibodies against IL-2, IL-10, and TGF-β1 to inhibit their function. Blocking IL-10 or TGF-β1 did not appear to decrease the percentage of Foxp3^{hi} cells. In contrast, blocking IL-2 abrogated T_{reg} induction **(Fig. 2.6A,B)**. This result supports the observation from other groups that IL-2 is important in Foxp3 induction, but challenges the observation from other groups that TGF-β1 is important in Foxp3 induction (49, 50). To verify that the blocking antibodies had sufficiently inhibited IL-10

and TGF-β1 in the cultures, we added Protein G to the supernates to remove the antibody-cytokine complexes and then assayed the cleared supernates using IL-10 and TGF-β1 ELISAs to determine if any free cytokine remained (see Protocol 5 in the Appendix for further detail). Using this method, IL-10 was effectively removed, but an average of 674 pg/mL TGF-β1 remained after removal of anti-TGF-β1/TGF-β1 complexes. Since some TGF-β1 remained, we can't eliminate the possibility that TGF-β1is involved in T_{reg} induction in our system. However, TGF-β1 was both secreted and surface expressed by cells costimulated through CD3+ICAM-1 and by cells costimulated through CD3+CD28. This indicates that costimulation through ICAM-1 provides unique signals leading to T_{reg} induction that costimulation through CD28 does not provide.

Signaling in induced T_{reg} cells and modulation of phenotype

To begin to identify some of the signaling processes occurring after costimulation through ICAM-1 that might lead to T_{reg} induction, we used intracellular flow cytometry to detect the activation status of p44/42 MAPK Erk1/2. Anti-phospho-p44/42MAPK-AlexaFluor488 antibody detects phosphorylation of MAPK residues Thr202 and Tyr204. MAPK phosphorylation can occur after many cellular events including TCR stimulation and cytokine stimulation to effect T cell activation and differentiation (51). A recent paper showed increased MAPK phosphorylation in activated T_{reg} cells (52). MAPK is activated after IL-2 signaling (50) and TCR stimulation, which are two events necessary for T_{reg} induction after ICAM-1 costimulation (**Fig. 2.2A and Fig. 2.6**). In addition, former graduate student Dr. Chintana Chirathaworn observed that ICAM-1 could co-localize with MAPK in immunoprecipitation experiments, suggesting it might be involved in ICAM-1 related activation or differentiation pathways (53). We observed that both costimulation through CD3+ICAM-1 and costimulation

through CD3+CD28 lead to phosphorylation of p44/42 MAPK, even several days after activation (**Fig. 2.7A**). When T_{reg} cells induced by costimulation through CD3+ICAM-1 were isolated using magnetic selection columns, the percentage of cells with activated p44/42 MAPK was increased compared to the total population of cells costimulated through CD3+ICAM-1 (**Fig. 2.7A,B**). This does not provide evidence that MAPK activation is involved in T_{reg} induction specifically, but it does show that the differentiated T_{reg} cells were using the MAPK pathway in some cellular process.

Other groups have shown that addition of retinoic acid can lead to the induction of T_{reg} cells and the upregulation of gut homing markers CCR9 and LPAM-1 (integrin α4β7), and that specialized dendritic cells in the GALT (gut-associated lymphoid tissue) can produce retinoic acid to help convert naïve cells to T_{reg} cells (54-57). *In vivo*, this is thought to be an important mechanism of mucosal tolerance. To determine if addition of retinoic acid could modulate T_{reg} induction or phenotype, we added 1-10 nM all-trans retinoic acid to stimulated cultures. Unlike other experiments presented in this chapter, we isolated naïve CD4+ T cells from tonsil tissue instead of peripheral blood for this experiment. In contrast to published data, we in general did not see an increase in T_{reg} induction after addition of retinoic acid. However, the GALT homing marker LPAM-1 (integrin αa4β7) was increased on both non-T_{reg} cells and most notably on $Foxp3^{hi}$ cells (**Fig. 2.8**). Since this experiment was done *in vitro*, one can only speculate that increased levels of LPAM-1 might allow these cells to remain in the gut environment *in vivo*.

T_{reg} cells induced after ICAM-1 costimulation have suppressor function

To verify that the CD4+CD25+$Foxp3^{hi}$ population generated after ICAM-1 costimulation could function as T_{reg} cells, we tested whether these cells could suppress the activation and proliferation of other T cells in an *in vitro* suppression assay (**Fig. 2.9A**). We first isolated naïve

CD4+ T cells and stimulated them using anti-CD3 plus anti-ICAM-1. After 10 days of stimulation, the stimulated cells were collected and separated into CD4+CD25+ and CD4+CD25(-) populations. These cells were labeled with a membrane-binding fluorescent dye, PKH26, to allow them to be gated out of the flow cytometry profile. This allowed us to analyze proliferation of the responder cells only. Also on this day, total T cells were collected from the peripheral blood of the original donor. A group of these total T cells was labeled with PKH26 to use as control cells. The remaining cells were labeled with the intracellular dye CFSE. Thus, the populations of cells used in the suppression assay were: 1) CFSE-labeled Responder T cells (newly isolated), 2) PKH26-labeled Control T cells (newly isolated), 3) PKH26-labeled CD4+CD25+ T_{reg} cells (from stimulated cultures), and 4) PKH26-labeled Control CD4+CD25(-) cells (from stimulated cultures). The cells were cultured at T_{reg} (or Control): Responder cell ratios of 1:4, 1:2 and 1:1 and stimulated with anti-CD3 plus anti-CD28 for 5 days. Proliferation of the CFSE-labeled responder cell population was measured by flow cytometry after gating-out the PKH26-labeled T_{reg} or control cells.

When the CD4+CD25+ cells that had differentiated in cultures costimulated through ICAM-1 were added to the responder cells, responder cell proliferation was greatly diminished**.** **Figure 2.9B** shows a suppression assay performed at a T_{reg}: Responder cell ratio of 1:4. In this experiment, only 15.4% of the responder cells divided when CD4+CD25+ T_{reg} cells were added to the culture and only one round of cell division occurred. In contrast, when responder cells only were measured or when control T cells or CD4+CD25(-) cells were added to the culture, over 50% of the responder cells divided and multiple rounds of cell division were observed. At each T_{reg}: Responder ratio, the CD4+CD25+ T_{reg} cells inhibited responder cell proliferation, and a dose-dependent effect was observed (**Fig. 2.9C**). The CD4+CD25(-) cells weakly inhibited

responder cell proliferation in some experiments. This type of response has been observed by other investigators (58), and could possibly be due to the presence of CD25(-) cells with suppressor function, or due to contamination of CD4+CD25+ cells in the CD4+CD25(-) population (purity of the CD4+CD25- population was >90%, data not shown). This experiment provides evidence that the cells exhibiting a T_{reg} phenotype can possess suppressive capabilities.

Figure 2.1. The initially purified cell population displayed a naïve phenotype. (A) Purified human naïve CD4+ T cells were CD45RA+ (left panel, cells stained with anti-CD45RA are shown in bold and cells stained with isotype control are shown in thin gray), CD45RO(-) (middle panel), and CD11a^{lo}CD27+ (right panel). The CD11a vs. CD27 plot was created after gating on CD45RA+ cells. Representative of greater than 10 experiments (left and right panels) or 6 experiments (middle panel). (B) Homing marker expression patterns on the naïve CD4+ T cells were CCR7+CD62L+ (left panel), integrin α4β7+ (middle panel), and integrin α4β1+ (right panel). Representative of 4 experiments. (C) Costimulatory molecule expression of ICAM-1 and CD28 on the initial naïve CD4+ T cell population. Representative of 1 experiment. (D) A small percentage of the naïve cells were weakly Foxp3+ (left panel, cells stained with anti-Foxp3 are shown in bold and cells stained with isotype control are shown in thin gray), and the cells are primarily CD25(-)CD127+ (right panel). Representative of greater than 10 experiments.

Figure 2.1

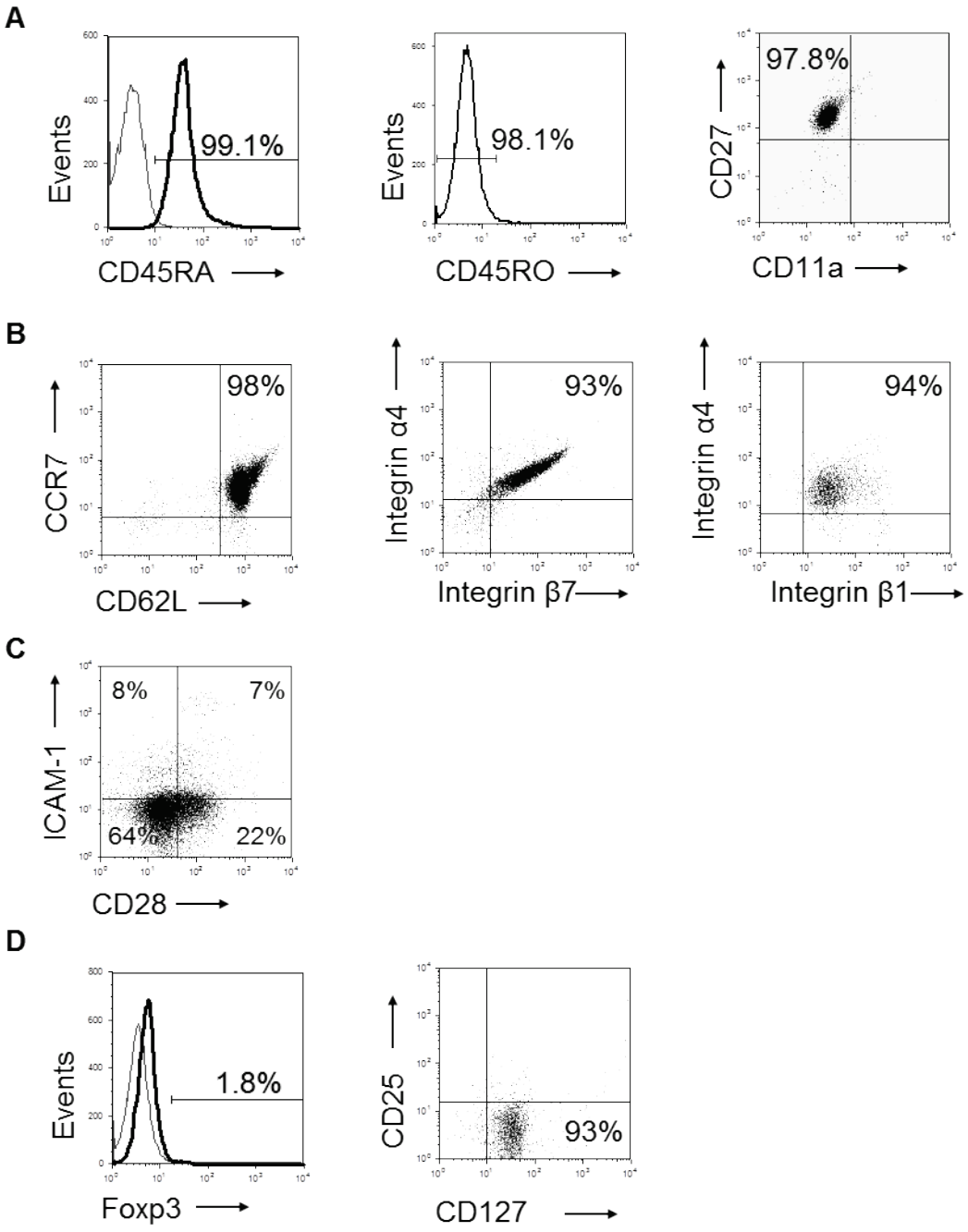

A
Events
99.1%
CD45RA
Events
98.1%
CD45RO
97.8%
CD27
CD11a
B
98%
CCR7
CD62L
Integrin α4
93%
Integrin β7
Integrin α4
94%
Integrin β1
C
8%
7%
64%
22%
ICAM-1
CD28
D
Events
1.8%
Foxp3
CD25
93%
CD127

Figure 2.2. T cells with a Foxp3+CD25+ T_{reg} phenotype were induced following costimulation of human naïve CD4+ T cells through ICAM-1, but not CD28. (A) Naïve CD4+ T cells were stimulated as indicated for 14 days and then analyzed by flow cytometry. The cells stained with anti-Foxp3 antibody are shown in bold whereas cells stained with isotype control are in thin gray. Representative of greater than 10 experiments. **(**B) Naïve CD4+ T cells were stimulated with anti-CD3 plus either anti-ICAM-1 or anti-CD28 and analyzed by flow cytometry for expression of CD25 at 7 days, CTLA-4 at 14 days, and CD127 or CD62L at 10 days of stimulation. Representative of greater than 10 experiments (CD25 or CD127 vs. Foxp3) or 3 experiments (CTLA-4 or CD62L vs. Foxp3).

Figure 2.2

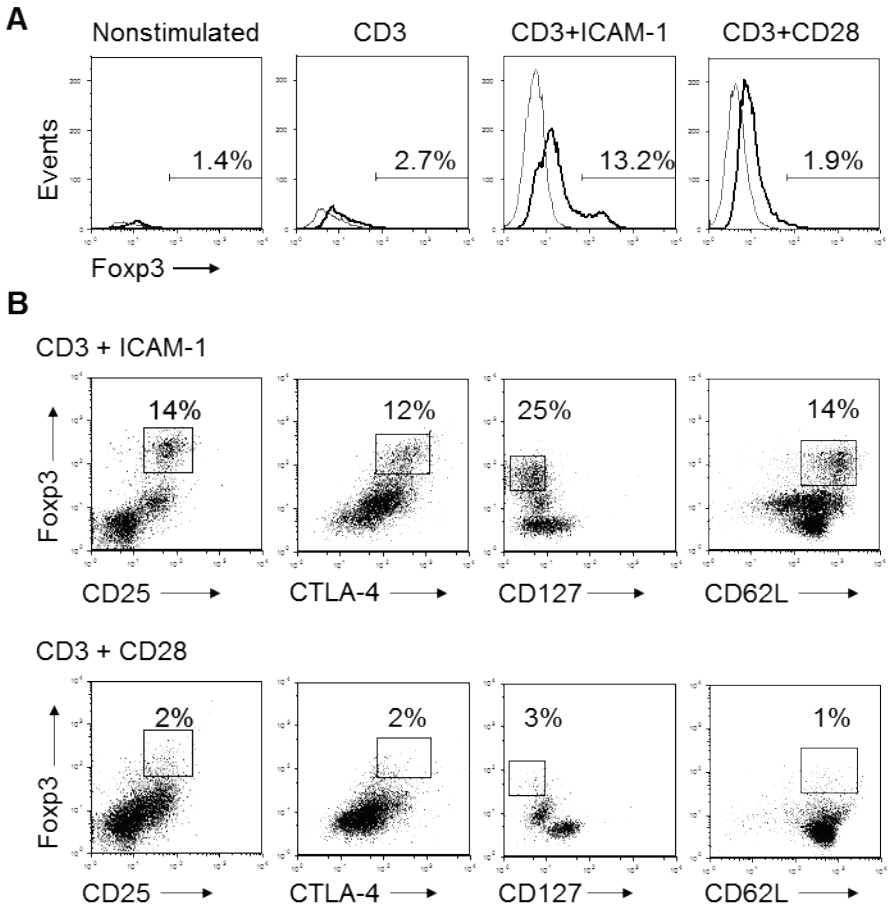

A
Nonstimulated
CD3
CD3+ICAM-1
CD3+CD28
Events
1.4%
2.7%
13.2%
1.9%
Foxp3
B
CD3 + ICAM-1
14%
12%
25%
14%
Foxp3
CD25
CTLA-4
CD127
CD62L
CD3 + CD28
2%
2%
3%
1%
Foxp3
CD25
CTLA-4
CD127
CD62L

Figure 2.3. Cells undergoing differentiation to T_{reg} cells were proliferative, and high levels of Foxp3 expression were maintained for at least 10 days. (A) Naïve CD4+ T cells were stained with CFSE and stimulated as indicated. Cell division and Foxp3 expression were analyzed after 7 days. Representative of 4 experiments. (B) Kinetics of Foxp3 induction were measured for cells stimulated through CD3 (hatched bars), CD3+ICAM-1 (closed bars), or CD3+CD28 (gray bars). The mean percentage of $Foxp3^{hi}$ cells in 5 separate experiments is shown for each time point +/- SEM. The asterisk indicates a statistically significant difference between the percentage of $Foxp3^{hi}$ cells after costimulation through ICAM-1 and the percentage of $Foxp3^{hi}$ cells after costimulation through CD28 (paired t-Test, one tail $p<0.05$). There were no other significant differences.

Figure 2.3

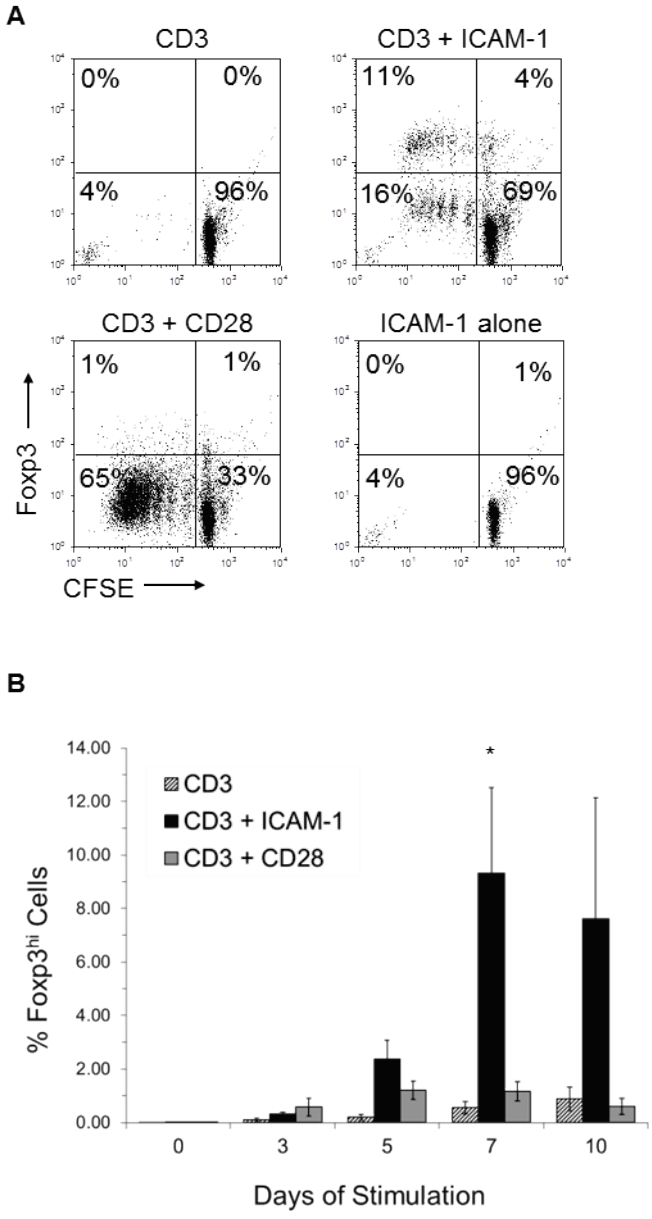

A
CD3
CD3 + ICAM-1
CD3 + CD28
ICAM-1 alone
0%
0%
4%
96%
11%
4%
16%
69%
1%
1%
65%
33%
0%
1%
4%
96%
Foxp3
CFSE
B
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
% Foxp3hi Cells
CD3
CD3 + ICAM-1
CD3 + CD28
*
0
3
5
7
10
Days of Stimulation

Figure 2.4. Costimulation through ICAM-1 enhances IL-10 secretion, but not TGF-β1 secretion when compared to costimulation through CD28. (A) Human naïve CD4+ T cells were stimulated with anti-CD3+anti-ICAM-1 or anti-CD3+anti-CD28. Cell culture supernates from the indicated times were assayed using IL-10 ELISA. Data are the means of duplicate samples from three experiments $\pm$ S.E.M. (B) Cell culture supernates from the indicated times were assayed using TGF-β1 ELISA. Data are the means of duplicate samples from four experiments $\pm$ S.E.M. The hatched bar indicates the mean concentration of TGF-β1 detected in the medium alone. The asterisk indicates a statistically significant difference between the concentration of TGF-β1 after costimulation through CD28 compared to the concentration of TGF-β1 after costimulation through ICAM-1 (paired t-Test, two tail $p<0.05$). There were no other significant differences. (C) Expression of LAP/membrane TGF-β1 (mTGF-β1) on the surface of naïve CD4+ T cells before stimulation (left panel) or after stimulation for 7 days (right panels). Representative of 5 experiments for Day 0 nonstimulated cells and representative of 2 experiments after stimulation.

Figure 2.4

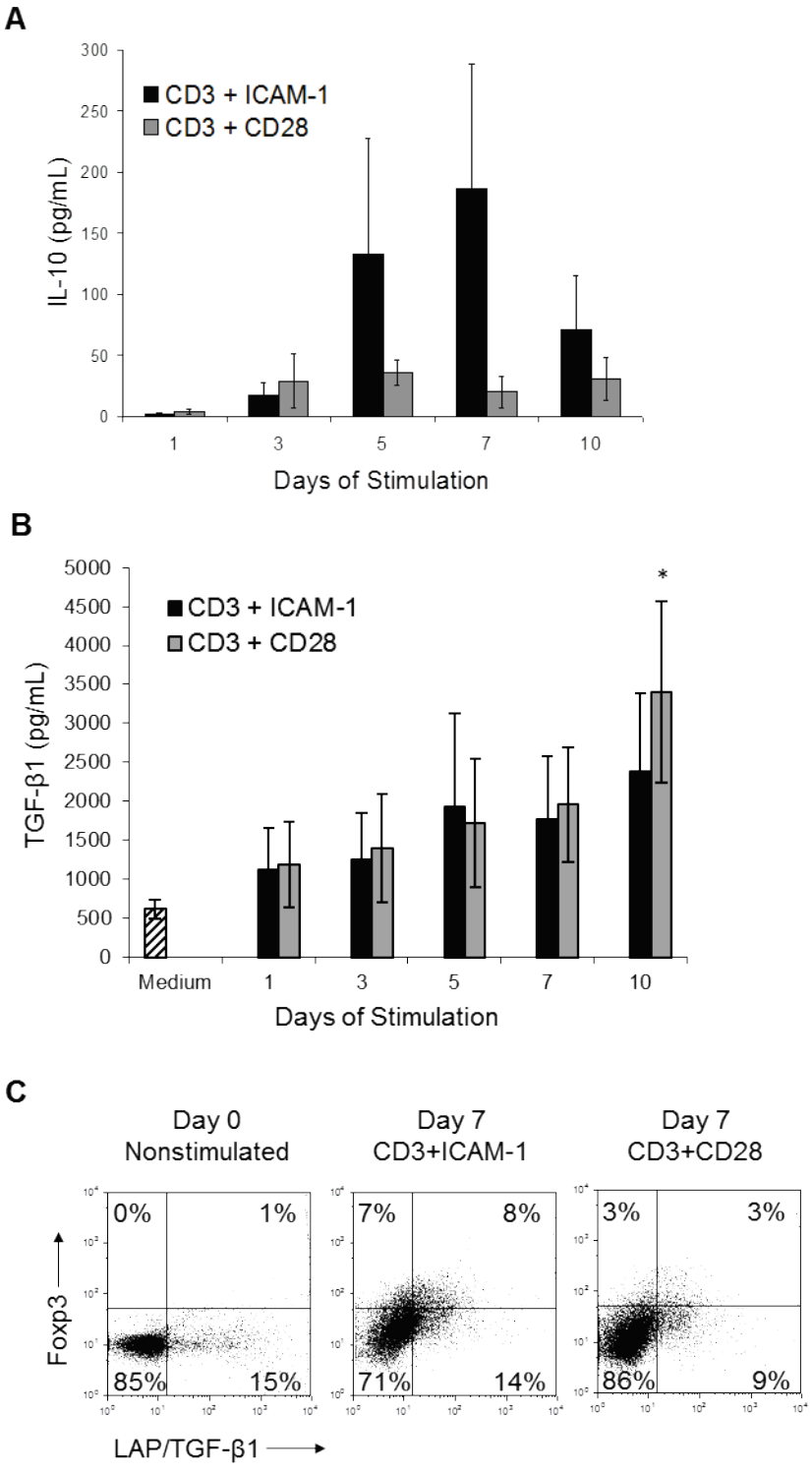

A
CD3 + ICAM-1
CD3 + CD28
IL-10 (pg/mL)
300
250
200
150
100
50
0
1
3
5
7
10
Days of Stimulation
B
CD3 + ICAM-1
CD3 + CD28
TGF-β1 (pg/mL)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
Medium
1
3
5
7
10
Days of Stimulation
C
Day 0
Nonstimulated
Day 7
CD3+ICAM-1
Day 7
CD3+CD28
0%
1%
85%
15%
7%
8%
71%
14%
3%
3%
86%
9%
Foxp3
LAP/TGF-β1

Figure 2.5. Addition of exogenous TGF-β1 plus IL-2 increases the percentage of naïve CD4+ T cells that differentiate after ICAM-1 costimulation. Naïve CD4+ T cells were stimulated for 7 days in the presence of exogenously added TGF-β1 (10 ng/mL) plus IL-2 (10 U/mL), and then analyzed for expression of Foxp3 and CD25. Representative of 2 experiments.

Figure 2.5

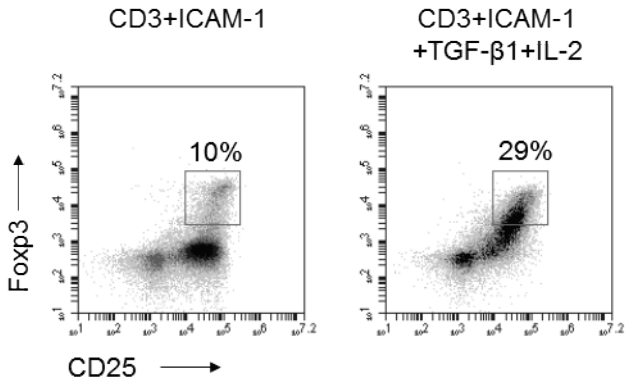


Figure 2.6. T_{reg} differentiation after ICAM-1 costimulation required IL-2, but not IL-10. (A) Human naïve CD4+ T cells were stimulated with anti-CD3+anti-ICAM-1 for 10 days in the presence of the blocking antibodies indicated. Blocking antibodies were added at 20 μg/ml on Day 0 and again on Day 5. Representative of 4 experiments. (B) Summary of the creation of $Foxp3^{hi}$ cells in the presence of cytokine-directed blocking antibody. Data are mean of 4 experiments $\pm$ SEM. The asterisk indicates a significant difference between sample with blocking antibody and sample without (paired t-Test, one tail $p \leq 0.05$).

Figure 2.6

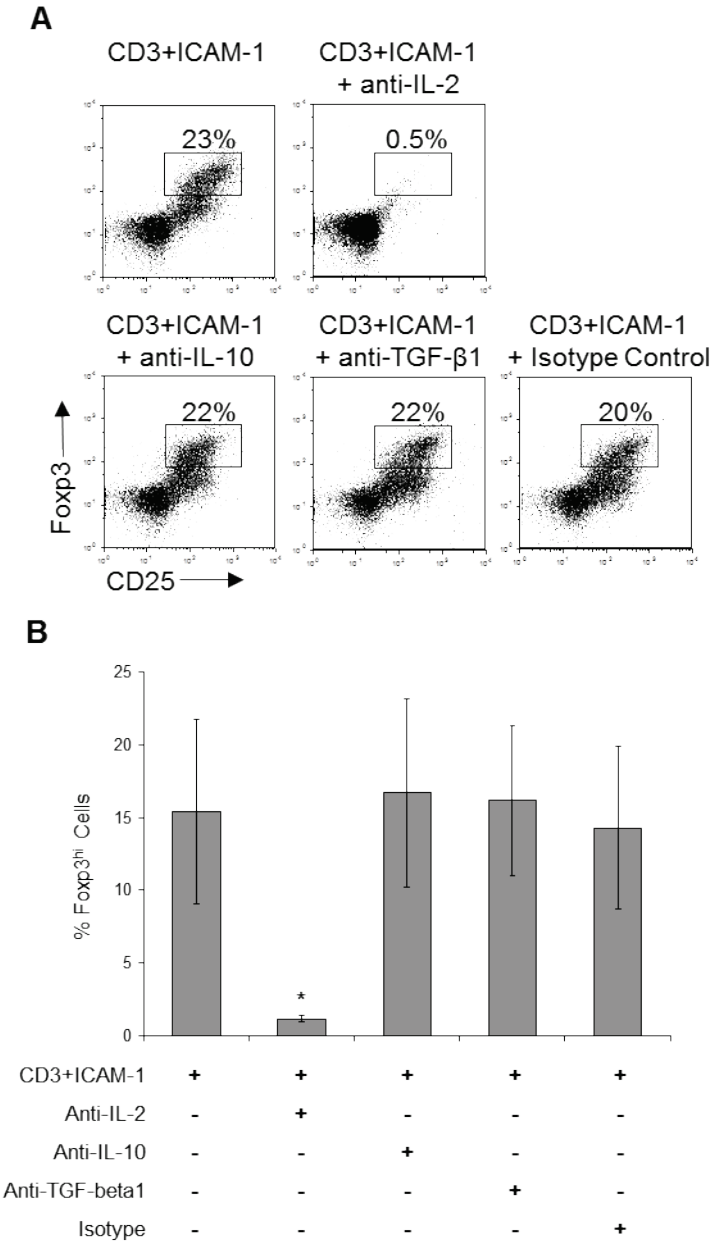

A
CD3+ICAM-1
CD3+ICAM-1
+ anti-IL-2
23%
0.5%
CD3+ICAM-1
+ anti-IL-10
CD3+ICAM-1
+ anti-TGF-β1
CD3+ICAM-1
+ Isotype Control
22%
22%
20%
Foxp3
CD25
B
% Foxp3hi Cells
25
20
15
10
5
0
*
CD3+ICAM-1 + + + + +
Anti-IL-2 - + - - -
Anti-IL-10 - - + - -
Anti-TGF-beta1 - - - + -
Isotype - - - - +

Figure 2.7. Costimulation through ICAM-1 leads to p44/42 MAPK Erk1/2 activation during the activation and differentiation process. (A) Intracellular phospho-p44/42 MAPK was detected on Day 13 of stimulation. Representative of 4 experiments performed on days 7-13 of stimulation. (B) In the same experiment shown in panel (A), CD127(-)CD25+ cells were isolated from the CD3+ICAM-1 stimulated cultures using magnetic separation after 13 days of stimulation. The bold black histograms indicate staining with anti-phospho-p44/42MAPK antibody and the thin grey histograms indicate staining with the isotype control antibody. Representative of 3 experiments performed on Days 7-13 of stimulation.

Figure 2.7

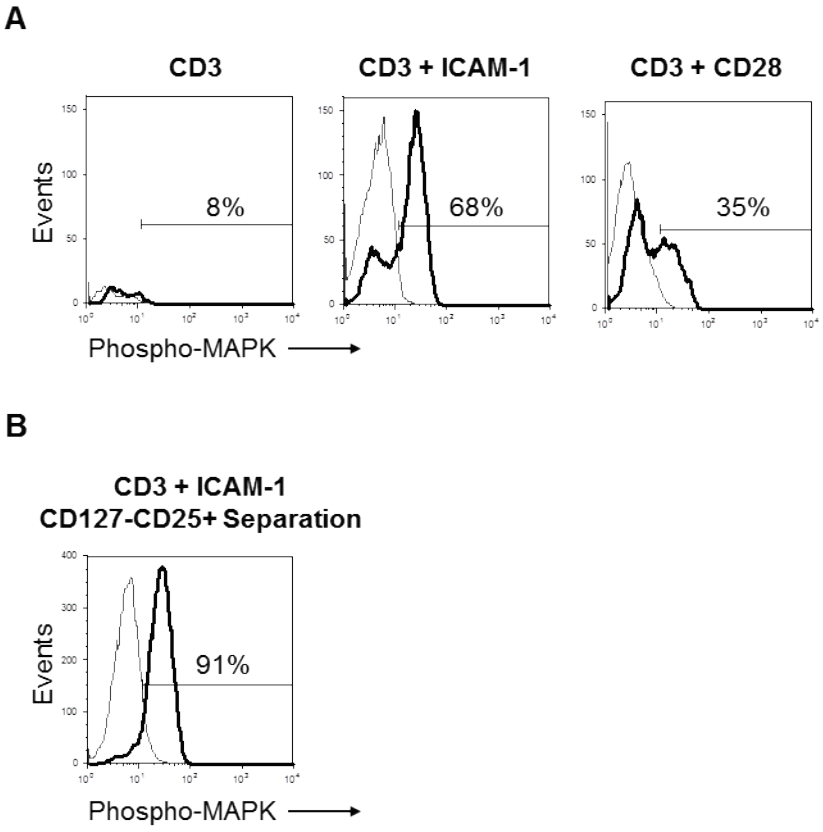

A
CD3
CD3 + ICAM-1
CD3 + CD28
Events
8%
68%
35%
Phospho-MAPK
B
CD3 + ICAM-1
CD127-CD25+ Separation
Events
91%
Phospho-MAPK

Figure 2.8. The GALT homing marker integrin α4β7 (LPAM-1, Lymphocyte Peyer's Patch Adhesion Molecule-1) can be upregulated upon addition of retinoic acid. Naïve CD4+ T cells were purified from tonsil tissue and stimulated with anti-CD3+anti-ICAM-1 with or without either 10 nM retinoic acid or DMSO control. The integrin α4β7 heterodimer and the T_{reg} marker Foxp3 were analyzed at day 8. The percentages and MFIs shown measure cells contained in the upper right quadrants only. Representative of 3 experiments using naïve CD4+ T cells from tonsil.

Figure 2.8

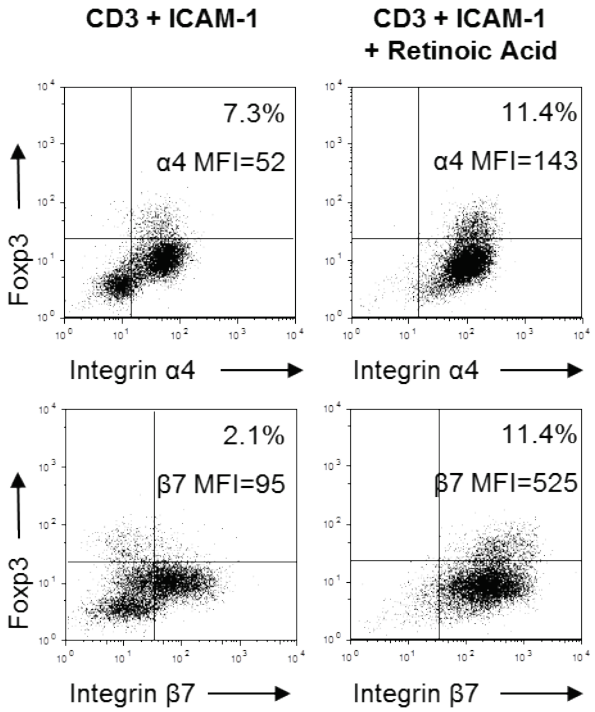
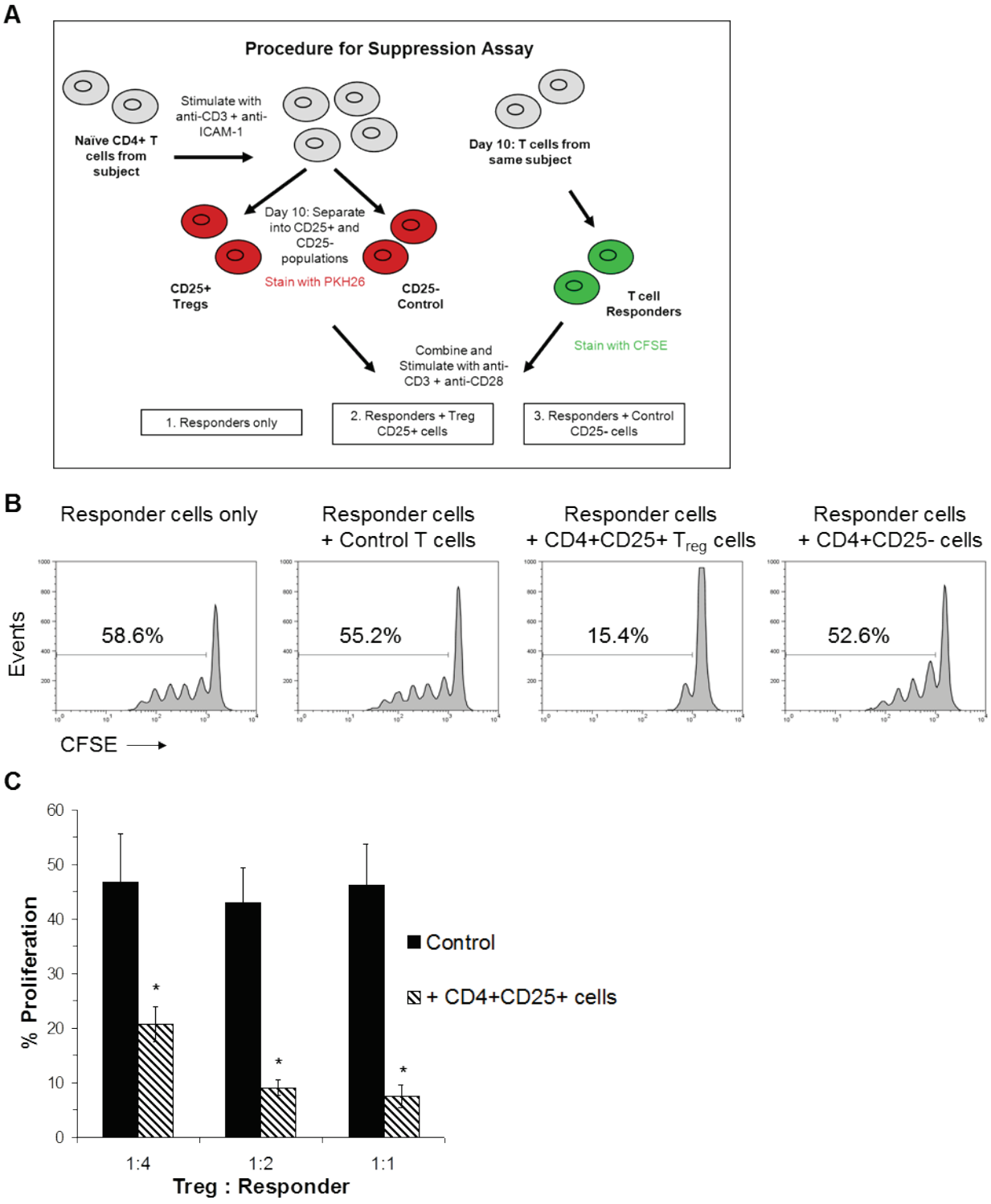

CD3 + ICAM-1
CD3 + ICAM-1
+ Retinoic Acid
7.3%
α4 MFI=52
11.4%
α4 MFI=143
Foxp3
Integrin α4
Integrin α4
2.1%
β7 MFI=95
11.4%
β7 MFI=525
Foxp3
Integrin β7
Integrin β7

Figure 2.9. CD4+CD25+ cells induced after costimulation through ICAM-1 suppressed responder cell proliferation. (A) Diagram of the experimental procedure. Human naïve CD4+ T cells were stimulated with anti-CD3+anti-ICAM-1 to induce differentiation to cells with a T_{reg} phenotype. After 10 days, cells were magnetically separated into CD4+CD25+ and CD4+CD25(-), and labeled with PKH26 dye to allow them to be gated out of the flow cytometry profile, facilitating analysis of proliferation by only responder cells. Also on day 10, total T cells to be used as responders were collected by a second bleed of the original donor. One aliquot of responder cells was labeled with PKH26 to use as control. The remaining cells were labeled with CFSE and used as responders. Thus, the populations of cells used in the suppression assay were: 1) CFSE-labeled Responder T cells (newly isolated), 2) PKH26-labeled Control T cells (newly isolated), 3) PKH26-labeled CD4+CD25+ T_{reg} cells (from stimulated cultures), and 4) PKH26-labeled CD4+CD25(-) cells (from stimulated cultures). The cells were cultured at T_{reg} (or Control): Responder cell ratios of 1:4, 1:2 and 1:1 and stimulated with anti-CD3+anti-CD28 for 5 days to induce proliferation. Proliferation of the responder cell population was measured by flow cytometry after gating-out the PKH26-labeled T_{reg} or control cells. (B) CD4+CD25+ T_{reg} cells [or control T cells or CD25(-) cells] at a T_{reg}: responder ratio of 1:4. The percentage of cells undergoing cell division is shown. Representative of 3 experiments. (C) Proliferation data from all three T_{reg}: responder ratios are presented for T_{reg} cells (CD4+CD25+, hatched bars) as well as the control T cells (closed bars). The data are shown as the mean % proliferation of responder cells from 3 separate experiments. Asterisks indicate statistically significant differences between control T cell samples and samples with added CD25+ cells (normalized to samples containing CFSE-labeled responder cells only, paired t-Test, one tail $p<0.05$).

Figure 2.9


A
Procedure for Suppression Assay
Naïve CD4+ T cells from subject
Stimulate with anti-CD3 + anti-ICAM-1
Day 10: T cells from same subject
Day 10: Separate into CD25+ and CD25- populations
Stain with PKH26
CD25+ Tregs
CD25- Control
T cell Responders
Stain with CFSE
Combine and Stimulate with anti-CD3 + anti-CD28
1. Responders only
2. Responders + Treg CD25+ cells
3. Responders + Control CD25- cells
B
Responder cells only
Responder cells + Control T cells
Responder cells + CD4+CD25+ Treg cells
Responder cells + CD4+CD25- cells
Events
58.6%
55.2%
15.4%
52.6%
CFSE
C
% Proliferation
Control
+ CD4+CD25+ cells
1:4
1:2
1:1
Treg : Responder

DISCUSSION

The data presented in this chapter suggest a previously unknown role for ICAM-1 in T_{reg} induction from human naïve precursors. Stimulation of human naïve CD4+ T cells (**Fig. 2.1**) through CD3+ICAM-1 generated a population of Foxp3+ CD25+ CTLA-4+ $CD127^{lo}$ cells (**Fig. 2.2A,B**). This phenotype corresponds to a T_{reg} phenotype described by other groups. It is notable that the T_{reg} cells induced after costimulation through ICAM-1 retain high expression of CD62L. Although our experiments were conducted *in vitro*, it is known that cells use CD62L to enter lymphoid tissues such as lymph nodes *in vivo*. Although it is speculative, the presence of CD62L on the surface of the T_{reg} cells that we detect might indicate that they would function as suppressor cells in lymphoid organs rather than at sites of inflammation. Our data indicate that the ICAM-1 induced T_{reg} cells are proliferative at some stage of differentiation (**Fig. 2.3A**). On average, we detect the highest percentage of T_{reg} cells at Day 7 of our stimulation cultures, although this varies among subjects (**Fig. 2.3B**). In contrast, cells with a T_{reg} phenotype were not generated by costimulation through CD3+CD28.

By assaying supernates from CD3+ICAM-1 and CD3+CD28 stimulated cultures, we detected more IL-10 secreted after ICAM-1 costimulation (**Fig. 2.4A**). Similar amounts of TGF-β1 were secreted after costimulation through either treatment, although mean TGF-β1 secretion was slightly higher on Day 10 of costimulation with CD3+CD28 (**Fig. 2.4B**). Cells stimulated through CD3+ICAM-1 and cells stimulated through CD3+CD28 expressed low levels of surface LAP/TGF-β1 (**Fig. 2.4C**). The addition of exogenous TGF-β1 plus IL-2, a method that is standard in the literature to induce T_{reg} cells, enhanced T_{reg} formation after costimulation through ICAM-1 (**Fig. 2.5**). This result might indicate that the IL-2 and/or TGF-β1 produced by cells costimulated though ICAM-1 might be involved in T_{reg} induction in our system. However, a

counter-argument is the result that CD3+CD28 stimulation also resulted in production of IL-2 and TGF-β1, without T_{reg} induction. Since we did not measure expression of cytokine receptors on the differentiating cells, we cannot determine if cells stimulated through CD3+ICAM-1 and cells stimulated through CD3+CD28 had the same ability to respond to cytokines present in the cultures. To further address the question of cytokine participation, we used blocking antibodies to attempt to inhibit the IL-2, TGF-β1, and IL-10 produced after CD3+ICAM-1 stimulation (**Fig. 2.6A,B**). Blocking these cytokines revealed that IL-2 was necessary for T_{reg} induction after costimulation through ICAM-1. Since TGF-β1 could only be partially blocked, it remains possible that it had a role in T_{reg} induction in our system.

Our results thus far indicated that signaling through the TCR/CD3 complex, ICAM-1, and IL-2R were important in differentiation of T_{reg} cells using our methods. To begin to ask what intracellular signaling cascades might be involved in either T_{reg} induction or function, we selected a signaling molecule involved in a multitude of T cell processes, p44/42 MAPK, and analyzed whether it was activated/phosporylated during the differentiation process in T_{reg} cells. We found that this molecule was activated in the majority of induced T_{reg} cells, suggesting that the MAPK signaling pathway might be involved in a process such as T_{reg} differentiation, survival, or function (**Fig. 2.7**). Addition of retinoic acid did not greatly increase the percentage of differentiating T_{reg} cells, but it did upregulate the GALT homing marker LPAM-1 (**Fig. 2.8**). Although this was an *in vitro* experiment, this result provides an example of how different factors in the microenvironment influence the resulting cellular phenotype. It may also demonstrate a way to program T_{reg} cells to home to certain sites in the body, which might be useful in T_{reg}-directed therapies. Finally, we determined that the ICAM-1 induced T_{reg} cells

could function as T_{reg} cells in an *in vitro* suppression assay by strongly inhibiting proliferation of autologous T cells (**Fig. 2.9B,C**).

Findings from other groups support a role for ICAM-1 in T_{reg} differentiation or function. Mouse and human regulatory T cells express higher levels of ICAM-1 than non-T_{reg} cells (59, 60). A more recent study showed that ICAM-$1^{-/-}$ mice have fewer T_{reg} cells in peripheral organs and an increased immune response *Mycobacterium tuberculosis* infection compared to wild-type mice (61). Although one study reported that ICAM-1 is not required for mouse CD25+ T_{reg} suppressor function (62), the role of ICAM-1 resident on human naïve cells in T_{reg} differentiation has not been investigated until now.

Many outcomes are possible from naive CD4+ T cell differentiation, including T_{H1} cells, T_{H2} cells, T_{H17} cells, memory cells, and T_{reg} cells. The specific tissue microenvironment that the naïve CD4+ T cell encounters will provide varying concentrations and types of cytokines, chemokines, and costimulatory molecule ligands that can polarize the differentiating T cell to specific lineages (63). On the other side of the equation, the differentiating T cell can respond differently to the cues in the microenvironment depending on the surface molecules that the T cell expresses. While dogma holds that naïve CD4+ T cells are a relatively homogenous population, new evidence suggests that several subpopulations actually exist (64). This may lead to differential naïve T cell responses to the same microenvironment.

Induction of T_{reg} cells after costimulation through ICAM-1 is consistent with what one would expect to be beneficial in controlling an immune response once that immune response is no longer needed. In CD3+ICAM-1 stimulated cultures, induction of T_{reg} cells is delayed for 5-7 days after stimulation. If we speculate and apply this observation to an *in vivo* scenario, the induced T_{reg} cells might function to suppress activated cells after an immune response has

already been initiated. In addition, signaling through ICAM-1 may provide an important sensing mechanism to the naïve T cell. While ICAM-1 is expressed on diverse cell types, the ICAM-1 ligands, LFA-1 (CD11a/CD18), Mac-1 (CD18/CD11b) and gp150/95 (CD18/CD11c) are expressed only on leukocytes. Also, for a strong interaction to occur between ICAM-1 and LFA-1, the heterodimers of LFA-1 must be in an activated conformation (65). Thus, signaling through ICAM-1 resident on a T cell may provide a mechanism by which the T cell can perceive that it is interacting with an activated leukocyte. Although it is speculative, one can imagine that a naïve CD4+ T cell in a lymphoid organ might encounter an activated APC both presenting cognate antigen and expressing ICAM-1 ligands in the active conformation which might lead to the differentiation outcome described in this chapter.

In summary, we have shown that stimulation of human naïve CD4+ T cells through CD3+ICAM-1 induced the differentiation of a population of T_{reg} cells. These cells expressed a T_{reg} phenotype as Foxp3+ CD25+ CTLA-4+ $CD127^{lo}$ and suppressed the activation and proliferation of responder T cells in co-culture experiments. These results suggest that costimulation through ICAM-1 might be worth further study for possible T_{reg}-directed therapies for immune conditions such as autoimmune diseases, allergies and asthma, and organ-transplant tolerance. One proposed treatment methodology might be to obtain blood from a patient, induce T_{reg} cells in culture by costimulating through ICAM-1, purify the T_{reg} population, and reintroduce the T_{reg} cells back into the same patient to initiate immune tolerance.

CHAPTER 2 ACKNOWLEDGEMENTS

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CHAPTER 3

AGING MAY AFFECT NAÏVE CD4+ T CELL DIFFERENTIATION TO REGULATORY T CELLS

ABSTRACT

Aging is accompanied by immunosenescence, the loss of proper immune system function. Due to thymic involution, naïve T cell development is gradually diminished. T cell activation and differentiation are also modified in older individuals. These age-related changes are expected to weaken immune responses to infections and to vaccination, and to increase the likelihood of cancer development. In this chapter, we compare costimulation of naïve CD4+ T cells obtained from the peripheral blood of older individuals (65 years of age or older) with naïve CD4+ T cells obtained from the peripheral blood of younger individuals (under age 65, typically ages 20-30 in our study). Unlike data obtained from younger individuals, costimulation of naïve CD4+ T cells from older individuals through ICAM-1 did not commonly lead to induction of a population of $Foxp3^{hi}$ regulatory T (T_{reg}) cells. These results support existing data that changes in T cell differentiation may occur during the process of aging.

NOTES

Some of the work presented in this chapter is included in a paper in preparation (Dotson *et al*.).

INTRODUCTION

Advances in medicine and public health have greatly improved the average life expectancy in the U.S., from 47.3 years for a child born in 1900 to 76.9 years for a child born in 2000 (1). As we age, changes take place on genetic, biochemical, and cellular levels that can lead to functional decline and subsequent health problems (2). Alterations can occur due to events such as DNA damage, telomere shortening, oxidative stress, altered protein glycation, or decreased proteasome function (3). Many age-related changes occur in the immune system, with consequences that include an increased risk for infections, cancers, and some inflammatory and autoimmune diseases. As one example of how aging affects the immune response, statistics show that 80-90% of deaths related to influenza virus infection occur in older individuals (4).

Many cells of the immune system and stages of the immune response undergo remodeling during the aging process. During hematopoiesis, the number of hematopoietic stem cells (HSCs) decreases with age in humans and most mouse strains studied, except for C57Bl/6 mice (5). Also, hematopoiesis favors myeloid over lymphoid development in older individuals (6). The thymus begins to involute about a year after birth and gradually becomes smaller with age, with a concomitant decrease in naïve T cell output (7). While naïve T cells continue to be produced by the thymus in aged mice, in contrast, adult human naïve T cells are mostly generated by proliferation of existing naïve T cells in the periphery (8). The percentages of naïve CD4+ and naïve CD8+ T cells in peripheral blood decreases, with concurrent increases in the percentage of memory T cells (9). Changes also occur in T cell function in older individuals. In naïve CD4+ T cells, there is poor immunological synapse formation which leads to decreased intracellular signaling after TCR triggering. This in turn leads to decreased T cell proliferation, decreased IL-2 production, and decreased cytokine help to B cells (3). CD8+ T cells exhibit

decreased cytotoxic responses in older individuals. A substantial percentage of T cells, especially within the CD8+ subset, down-regulate the costimulatory molecule CD28, most likely due to chronic antigenic stimulation (10). Both CD8+ and CD4+ T cells can undergo clonal expansion, leading to decreased TCR diversity (11). A sizeable percentage of these clonally expanded cells is specific for cytomegalovirus (CMV) (12). The clonally expanded cells are proposed to take up space in secondary lymphoid tissues, leaving less room for the naïve T cells that emigrate from the thymus and contributing to a reduced naïve/memory T cell ratio compared to younger individuals (12, 13). Cytokine profiles also have been shown to change with aging, with T_{h2} responses favored over T_{h1} responses (3).

Previously published data describing age-related changes in T_{reg} cell numbers and function have been inconsistent. Some studies suggest that $Foxp3^{hi}$ T_{reg} cell percentages slightly but statistically significantly increase with aging, both in human peripheral blood and C57Bl/6 mouse tissues, but not in mouse peripheral blood (14, 15). However, other investigators reported that $Foxp3^{hi}$ T_{reg} cell percentages were similar between younger and older individuals (16). One review compared 14 studies that analyzed the percentage of $CD4+CD25^{hi}$ T_{reg} cells in human blood, and only 3 of these studies found that the percentage of T_{reg} cells correlated with age (17). Some reports have shown that T_{reg} cells in older individuals still function well as suppressor cells and have suggested that they may contribute to decreased immune responses seen in older individuals. For example, mouse studies have suggested that T_{reg} cells can impair anti-tumor responses in aged Balb/c mice (18), can influence the reactivation of chronic infections (14), and can increase in percentage in aged mice during influenza virus infection (19). *In vitro* experiments using cells from humans (16) or C57Bl/6 and Balb/c mice (19) showed no age-related changes in T_{reg}-mediated suppression of T cell proliferation. However, a Balb/c mouse

study showed age-related T_{reg} defects in some suppressor functions such as preventing cytokine production and decreasing DTH responses (20). (Depending on the study, young mice were between 2-6 months of age, and aged mice were between 20-28 months of age.) Taken together, the existing literature connecting T_{reg} cells and aging has been inconsistent.

Seeming to contradict the decreased immune responses described in older individuals, there is also an increased occurrence of some inflammatory and autoimmune diseases with aging. Older individuals have increased production of inflammatory cytokines such as IL-6 and TNF-α and increased numbers of T_{h17} cells (3, 11). Some diseases associated with aging such as atherosclerosis and Alzheimer's disease are recognized to have inflammatory components (3). Autoantibody production has been reported to be increased in older individuals, and some autoimmune diseases such as Hashimoto's thyroiditis, Sjogren's disease, and pulmonary fibrosis can have a late-onset (3, 21). Another connection between aging and autoimmunity is evidence that the immune systems of patients with autoimmune diseases show premature aging characteristics such as fewer naïve T cells and T cells with shortened telomeres (10). The precise role of T_{reg} cells in late-onset autoimmunity and inflammatory disease is still to be determined.

In this chapter, we asked if ICAM-1 could provide a costimulatory signal in naïve CD4+ T cells from older individuals in a similar manner to our results from younger individuals using our *in vitro* culture system. We found some similarities and some differences between groups when analyzing differentiation outcome.

MATERIALS AND METHODS

Cell Purification

Naïve CD4+ T cells were isolated from the peripheral blood of healthy donors using the same procedure described in the Materials and Methods in Chapter 2 and Appendix Protocol 1. Naïve CD4+ T cells for this study were defined as CD45RA+CD11a^{lo}CD27+ or CD45RO(-) CD11a^{lo}CD27+.

Cell Culture Reagents

Cell culture reagents used in this chapter are the same as those described in the Chapter 2 Materials and Methods.

Addition of Exogenous Cytokines

In most experiments, no exogenous cytokines were added to the cell cultures. However, in one experiment, where indicated, we added recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) and IL-2 (Boehringer Mannheim/Roche, Mannheim, Germany) as described in the Chapter 2 Materials and Methods. TGF-β1 was used at 10 ng/mL and IL-2 was used at 10 U/mL.

Stimulating Antibodies

The antibody clones, antibody concentrations, method of adhering antibodies to the tissue-culture plates, and method of antibody-mediated stimulation of cells used in this chapter are the same as those described in the Chapter 2 Materials and Methods and Appendix Protocol 3.

Flow Cytometry Antibodies

Information for most flow cytometry antibodies used in this chapter is listed in the Chapter 2 Materials and Methods. Antibodies used in this chapter, but not listed in Chapter 2 are anti-CD4-FITC and anti-CD4-TriColor which were from Caltag/Life Technologies (Carlsbad, CA).

Flow Cytometry Surface Staining

The flow cytometry staining procedure to detect cell-surface proteins is described in the Chapter 2 Materials and Methods and Appendix Protocol 4.

Intracellular Flow Cytometry Staining for Foxp3

The flow cytometry staining procedure to detect intracellular Foxp3 protein followed the protocol outlined by the eBioscience technical sheet, with minor modifications described in the Materials and Methods in Chapter 2.

Flow Cytometry Analysis

Flow cytometry and data analysis were performed as described in the Chapter 2 Materials and Methods.

Cytokine ELISA

The concentration of TGF-β1 secreted by stimulated cells was analyzed by ELISA using Human TGF-β1 Quantikine kits (R&D Systems) as described in the Chapter 2 Materials and Methods.

Statistical Analysis

Statistical significance was determined using either t-Tests or One-way ANOVA to compare the groups indicated. Differences were considered statistically significant if $p < 0.05$. Data shown in graphs were analyzed by either Microsoft Excel (Redmond, WA) or GraphPad Prism (La Jolla, CA).

Human Subjects

Peripheral blood was obtained after informed consent of healthy volunteers. Procedures were approved by The University of Kansas Institutional Review Board. Subjects were categorized as "older individuals" if they were 65 years of age or older, and "younger individuals" if they were less than 65 years of age. The older individuals in our study were 8 men between the ages of 67 to 81 years at the time of blood donation, with an average age of 74 years in the 14 experiments performed. Younger individuals were typically between the ages of 20 to 30, and included both men and women.

RESULTS

Cells purified from older and younger subjects had similar expression of the proteins studied

To begin to examine how aging might affect naïve CD4+ T cell activation and differentiation, we isolated naïve CD4+ T cells from the peripheral blood of healthy older individuals and compared the responses of these cells to results previously obtained with naïve CD4+ T cells from younger subjects (22, 23). In particular, I was interested in determining whether naïve CD4+ T cells from older subjects could differentiate to Foxp3^{hi} T_{reg} cells in a similar manner to our studies in younger individuals described in Chapter 2.

We first analyzed the native percentage of Foxp3+ cells in the total CD4+ population in PBMCs from older individuals. As mentioned in the Introduction to this chapter, previously published data on this subject have been inconsistent. We detected Foxp3+ cells in the CD4+ population, and found that the percentage of Foxp3+ cells was similar between groups of older individuals and younger individuals in the absence of stimulation. The difference between groups was slight and not statistically significant (**Fig. 3.1A,B**).

Next, we analyzed the phenotype of the naïve CD4+ T cells isolated from older individuals and compared them to the equivalent population from younger individuals. Even though the thymus begins to involute around one year of age and becomes progressively smaller over time, older individuals still have a small amount of functional thymus and some naïve T cells (7). The phenotype of the naïve CD4+ T cells purified using the StemSep Human Naïve CD4+ T Cell Enrichment Kit was similar between older and younger subjects for the protein markers we analyzed (**Fig. 3.2A,B,C** and Fig. 2.1). Naïve CD4+ T cells from both older and younger individuals expressed CD45RA, which is the isoform of CD45 expressed on naïve T cells (**Fig. 3.2A**). Also like younger individuals, older individuals had naïve CD4+ T cells that

expressed high levels of the selectin CD62L (**Fig. 3.2B**), and low but detectable levels of the costimulatory molecules ICAM-1 and CD28 (**Fig. 3.2C**). It should be noted that the data in **Figure 3.2** are preliminary since only 1 experiment was performed in some cases. However, another graduate student in our lab, Abby Dotson, also obtained data that support the results shown in **Figure 3.2** (data not shown). Also of note, the purity and yield of naïve CD4+ T cells was sometimes lower for older individuals, although this was variable among subjects (data not shown). When experiments were conducted with a less pure naïve population, there may have been error associated with contaminating non-naive cells.

Corresponding to results from younger individuals (Fig. 2.1), there was a small population of $Foxp3^{lo}$ cells in the newly isolated naïve CD4+ T cell population from older individuals (**Fig. 3.3A**). The profile of CD127 (IL-7Rα) and CD25 (IL-2Rα) expression was also similar between groups (**Fig. 3.3B**). The results presented in **Fig. 3.2 and 3.3** suggest that the naïve CD4+ T cells are phenotypically similar, at least for the limited number of proteins we studied.

Some costimulation results were similar between groups, while others differed

Using our *in vitro* cell culture system, we next studied whether activation and differentiation to functional cells might be altered by aging. The literature suggests that TCR stimulation is often impaired in T cells from older individuals (3). Naïve CD4+ T cells from older individuals were stimulated using the methods described in Chapter 2. Antibodies against CD3, CD3+ICAM-1, CD3+LFA-1 (CD11a), and CD3+CD28 were used as agents to induce stimulation. While the initial phenotype of naïve T cells appeared similar between the older and younger subjects studied, the cells sometimes differed in how they responded to stimulation.

An important outcome of costimulation that we studied was cellular proliferation. Classic costimulatory molecules enhance T cell proliferation and survival after activation. Abby Dotson analyzed whether naïve CD4+ T cells treated with various stimuli could expand in number in culture. Cell numbers were counted by adding flow cytometry counting beads to cell samples and analyzing samples using a BD FACScan. Over the course of 14 days of stimulation, cell numbers in culture increased after costimulation through ICAM-1 or LFA-1, but decreased after stimulation through CD3 alone or costimulation through CD28 (data not shown, are presented in Abby Dotson's dissertation). These data suggest that proliferation was occurring after costimulation through ICAM-1 and LFA-1. Proliferation could also have been occurring after stimulation through CD3 alone or costimulation through CD28, but these results could have been masked if there was concurrent cell death. These data contrast with data previously obtained from younger individuals in which costimulation through both ICAM-1 and CD28 increased cell numbers, while costimulation through LFA-1 did not, although data were only presented to Day 7 of stimulation in our lab's previous study (22). Further studies using CFSE as a cell proliferation dye and Annexin V and 7-AAD as cell death tracking reagents will help to further clarify how well cells are proliferating and surviving.

Next, we analyzed the ability of naïve CD4+ T cells from older individuals to differentiate to various cell types. My project focused on differentiation to T_{reg} cells, while Abby's project focused on differentiation to effector and memory cells. Data showing that either CD3+ICAM-1 or CD3+CD28 stimulation of naïve CD4+ T cells from younger individuals can generate a memory population were previously published by our lab (22). Some of Abby's data on effector and memory differentiation from naïve CD4+ T cells from older individuals are shown in **Figure 3.4**. After 14 days in culture, both costimulation through ICAM-1 and through

CD28 yielded populations with phenotypes of effector cells [CD45RA(-) and CD11a^{hi}CD27+ within the CD45RA(-) gate] and memory cells [CD45RA(-) and CD11a^{hi}CD27(-) within the CD45RA(-) gate]. Cells costimulated through LFA-1 (CD11a) did not appear to up-regulate CD11a, and therefore did not display either a typical effector or memory phenotype. Even though the mean percentage of memory cells at Day 14 of stimulation was similar between CD3+ICAM-1 and CD3+CD28 stimulation, the mean number of memory cells per cell culture well was higher after CD3+ICAM-1 stimulation than after CD3+CD28 stimulation (data not shown, data are presented in Abby Dotson's dissertation). These data suggest that while naïve CD4+ T cells from older individuals were able to differentiate to memory cells after costimulation through CD28, they perhaps did not proliferate as well or survive as well as naïve CD4+ T cells costimulated through ICAM-1.

My project was to determine if naïve CD4+ T cells from older individuals could differentiate to Foxp3^{hi} T_{reg} cells in the same manner as cells from younger individuals. Cells were stimulated for various time-points (range of 3-14 days, most often analyzed at 7 days) and then analyzed for the expression of T_{reg} markers Foxp3 and CD25. In contrast to results obtained using naïve CD4+ T cells from younger individuals (Fig. 2.2B) (23), naïve CD4+ T cells from older individuals did not routinely differentiate to Foxp3^{hi} cells (**Fig. 3.5A,B**). We conducted experiments using naïve CD4+ T cells obtained from 7 older subjects. Out of 9 experiments performed, 7 experiments showed that the naïve cells from older individuals did not differentiate to a population of Foxp3^{hi} cells. However, in 2 experiments we detected a Foxp3^{hi} population when naïve cells from older individuals were stimulated. In 1 of these experiments we detected approximately 12% Foxp3^{hi} cells after costimulation through ICAM-1 and approximately 11% of Foxp3^{hi} cells after costimulation through CD28 on Day 7 of stimulation, and in 1 experiment we

detected approximately 8% Foxp3^{hi} cells after costimulation through CD28 on Day 7 of stimulation. Results from the experiments performed on Day 7 of stimulation are averaged in **Figure 3.5B** for groups of both older and younger individuals. These data indicate that, in most experiments, naïve CD4+ T cells from older individuals did not differentiate to a T_{reg} phenotype in the same manner as naïve CD4+ T cells from younger individuals after costimulation through ICAM-1. A CD25+ population with intermediate Foxp3 expression (Foxp3^{int}) was generated following stimulation of naïve CD4+ T cells from older individuals by each stimulation treatment (**Fig. 3.5A**). The phenotype of this population suggests that it most likely represents activated, non-T_{reg} cells.

To begin to understand why the naïve CD4+ T cells from older individuals did not typically differentiate to Foxp3^{hi} cells after costimulation through ICAM-1, we asked if the cells were also able to differentiate to Foxp3^{hi} cells using the established method of adding exogenous TGF-β1 plus IL-2 cytokines to stimulated cultures (24). Results from this experiment might suggest whether the cells were defective in a general T_{reg} differentiation process or were defective in differentiation specifically involving ICAM-1. When exogenous TGF-β1 plus IL-2 were added to the cultures, we observed enhanced differentiation of Foxp3^{hi} cells after both costimulation through ICAM-1 and CD28 (**Fig. 3.6**). Because differentiation after cytokine addition was observed after both costimulation treatments, we concluded that differentiation in this experiment was due to the presence of an activation signal plus T_{reg}-inducing cytokines. However, this experiment was only performed once, so repeat experiments would be required to support this conclusion. Also, the population of Foxp3^{hi} cells after CD3+CD28 costimulation without cytokine treatment was anomalous in this experiment.

We also began to study what cytokines the naïve CD4+ T cells from older individuals could secrete after costimulation. We were interested in TGF-β1 since it is an immunosuppressive cytokine, and it has been shown to induce T_{reg} differentiation *in vitro* (25). **Figure 3.7** shows TGF-β1 ELISA results using supernates collected after 7 days of stimulation. TGF-β1 was produced by the cells obtained from the two older subjects when cells were costimulated through either ICAM-1 or CD28. Although the TGF-β1 concentration varied greatly between the two older subjects tested, the mean TGF-β1 concentration was similar to results from younger individuals (Fig. 2.4). This indicates that although cells with a $Foxp3^{hi}$ T_{reg} phenotype did not differentiate, there were cells in cultures costimulated through either ICAM-1 or CD28 that could produce TGF-β1.

Summary of Differentiation Results

Results for our differentiation studies are summarized in **Table 3.1**. Naïve CD4+ T cells from older individuals could differentiate to cells with the phenotype of effector and memory, but usually not T_{reg} cells, after both costimulation through ICAM-1 or CD28. Naïve CD4+ T cells from younger individuals could differentiate to cells with the phenotype of effector, memory, and T_{reg} cells after costimulation though ICAM-1, but only effector and memory after costimulation through CD28.

Figure 3.1. (A) The PBMC populations isolated from older individuals and younger individuals contained similar percentages of Foxp3+ cells before stimulation. Newly purified PBMCs from younger and older subjects were stained for expression of CD4, CD25, and Foxp3. The Foxp3 vs. CD25 dot plots represent cells within the CD4+ gate. Representative of PBMCs from 6 older individuals and 5 younger individuals. (B) The mean percentage of CD4+ PBMCs that are Foxp3+. Data are the mean of 6 samples from older individuals and 5 samples from younger individuals ± SEM. Differences between older and younger individuals were not statistically significant.

Figure 3.1

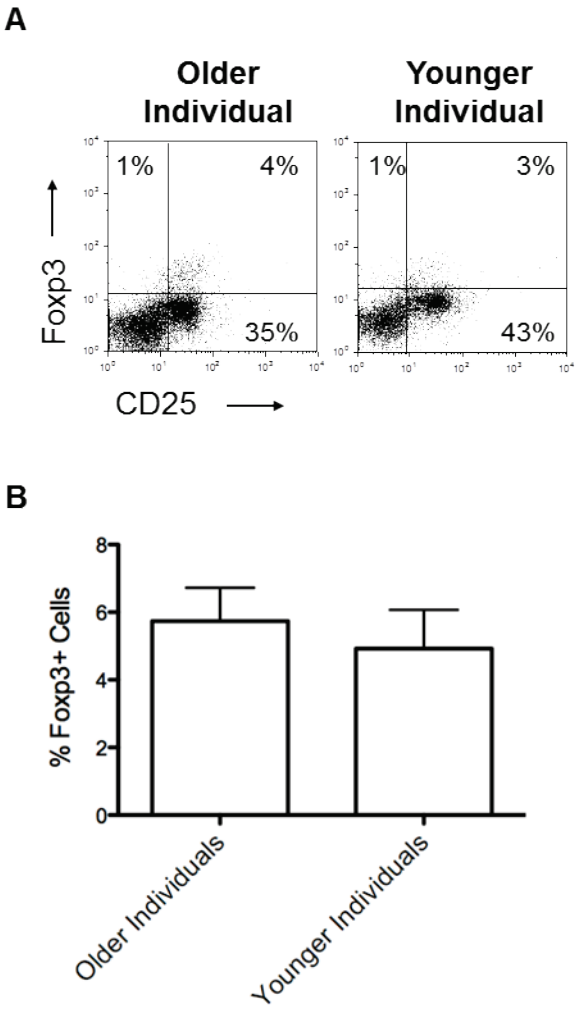
A
Older Individual
Younger Individual
1%
4%
35%
1%
3%
43%
Foxp3
CD25
B
% Foxp3+ Cells
0
2
4
6
8
Older Individuals
Younger Individuals

Figure 3.2. The newly purified naïve CD4+ T cell population appeared phenotypically similar in older and younger individuals. Data for younger individuals are the same as shown in Figure 2.1, or are adaptations of Figure 2.1. (A) The naïve CD4+ T cell population is CD45RA+. Representative of 1 experiment using cells from an older individual and greater than 10 experiments using cells from younger individuals. (B) The naïve CD4+ T cell population is CD62L+. Representative of 1 experiment using cells from an older individual and 4 experiments using cells from younger individuals. (C) The naïve CD4+ T cell population expresses low but detectable levels of ICAM-1 and CD28. Representative of 1 experiment using cells from an older individual and 1 experiment using cells from a younger individual.

Figure 3.2

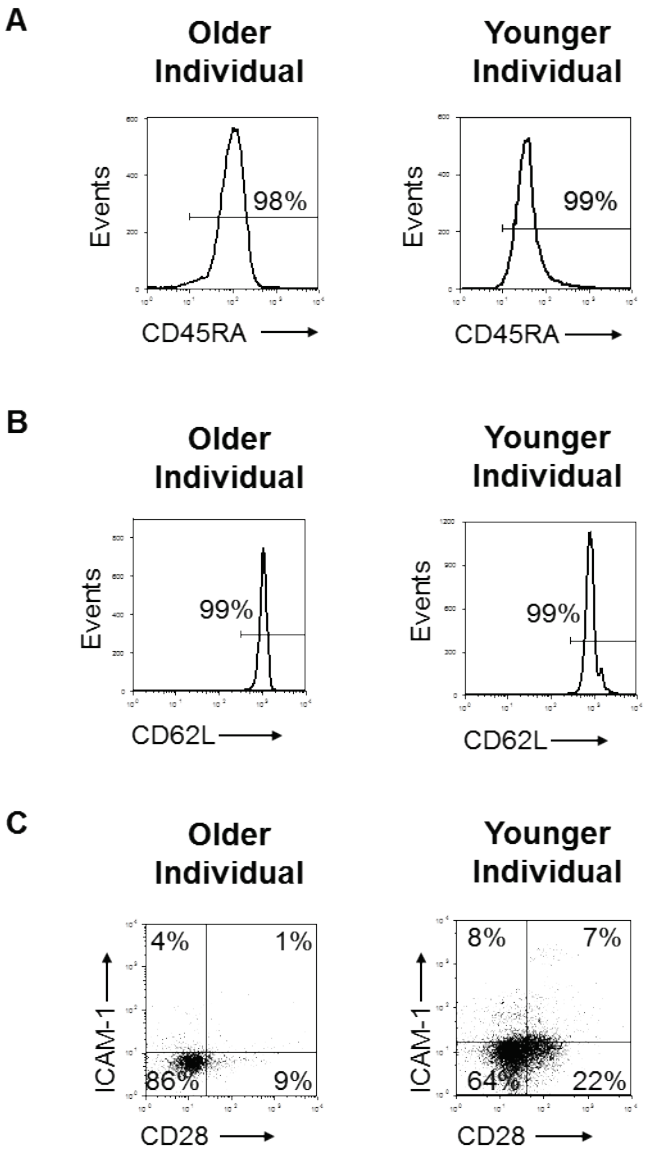
A
Older Individual
Younger Individual
Events
98%
99%
CD45RA
B
Older Individual
Younger Individual
Events
99%
99%
CD62L
C
Older Individual
Younger Individual
ICAM-1
4%
1%
86%
9%
8%
7%
64%
22%
CD28

Figure 3.3. A small percentage of the newly purified naïve CD4+ T cells from both older individuals and younger individuals is Foxp3^{lo}. Data for younger individuals are the same as shown in Figure 2.1. (A) A small percentage of the naïve cells was weakly Foxp3+. Representative of 8 experiments using cells from older individuals and greater than 10 experiments using cells from younger individuals. (B) The naïve CD4+ T cells were primarily CD25(-)CD127+. Representative of 2 experiments using cells from older individuals and greater than 10 experiments using cells from younger individuals.

Figure 3.3

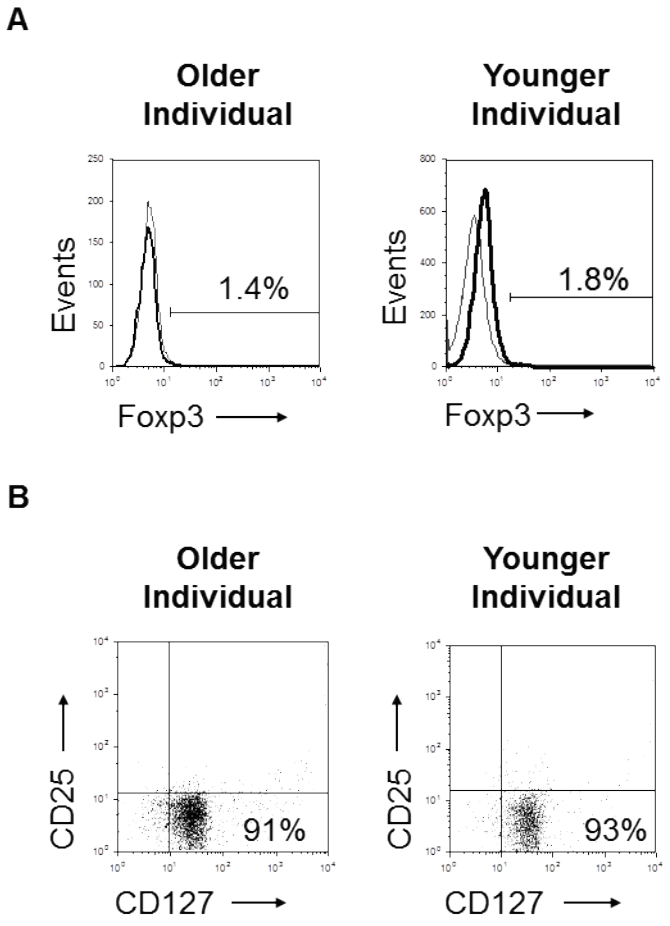
A
Older Individual
Younger Individual
Events
1.4%
1.8%
Foxp3
B
CD25
CD127
91%
93%

Figure 3.4. Naïve CD4+ T cells purified from older individuals can differentiate to effector and memory phenotypes after costimulation through either ICAM-1 or CD28. This figure shows data gathered by graduate student Abby Dotson, and is also presented in her dissertation and in a manuscript in preparation. Naïve CD4+ T cells from older individuals were stimulated for 14 days and then analyzed for expression of CD11a and CD27 after gating on either the CD45RA+ cells (upper left panel) or CD45RA(-) cells. In plots in which cells were gated on CD45RA(-) cells, effector cells are represented in the upper right quadrant, while memory cells are represented in the lower right quadrant. Representative of between 3-6 experiments (3 experiments for costimulation through LFA-1, and 6 experiments for costimulation through either ICAM-1 or CD28).

Figure 3.4

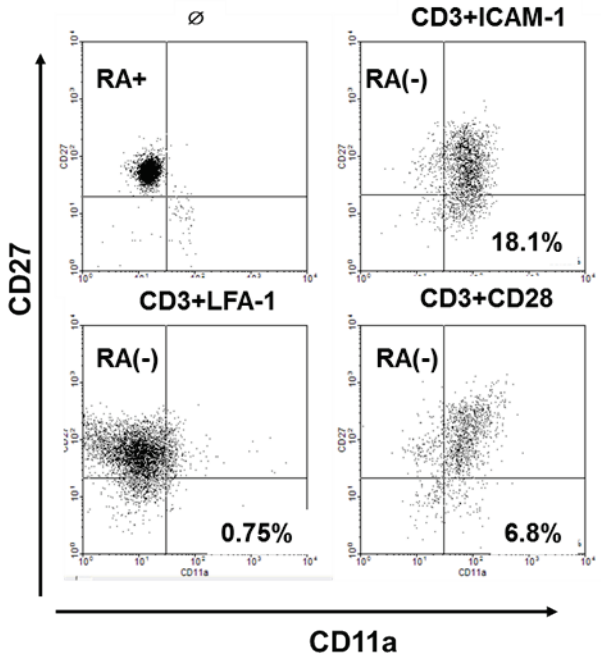

Figure 3.5. Naïve CD4+ T cells purified from older individuals and costimulated through ICAM-1 or CD28 do not routinely differentiate to Foxp3^{hi} cells. (A) Naïve CD4+ T cells from an older individual were stimulated as indicated for 7 days and then stained for the T_{reg} markers Foxp3 and CD25. Data from cells obtained from a younger individual are shown for comparison. Representative of 7 experiments for older individuals and greater than 10 experiments for younger individuals. Experiments were conducted using naïve CD4+ T cells from 7 different older subjects on Days 7-14 of stimulation in 9 total experiments. In experiments using cells from older subjects, 7 experiments gave negative results and 2 experiments gave a positive result for a Foxp3^{hi} population. (B) Comparison of induction of Foxp3^{hi} CD25+ cells using cells obtained from older or younger individuals. Data are the means of 4-6 experiments for cells from older individuals (4 experiments for CD3 stimulation and 6 experiments for both ICAM-1 and CD28 costimulation) and 7-11 experiments for cells from younger individuals (7 experiments for stimulation through CD3, 11 experiments for costimulation through ICAM-1 or CD28) on Day 7 of stimulation ± SEM. The asterisks indicate statistically significant differences between the percentage of Foxp3^{hi} CD25+ cells after stimulation of cells from younger individuals through CD3 and the percentage of Foxp3^{hi} CD25+ cells after stimulation of cells from younger individuals through CD3+ICAM-1 (One-way ANOVA with Tukey's Multiple Comparison post-test, ** $p<0.01$), and between the percentage of Foxp3^{hi} CD25+ cells after stimulation of cells from younger individuals through CD3+ICAM-1 and the percentage of Foxp3^{hi} CD25+ cells after stimulation of cells from younger individuals through CD3+CD28 (One-way ANOVA with Tukey's Multiple Comparison post-test, * $p<0.05$). There were no other significant differences.

Figure 3.5

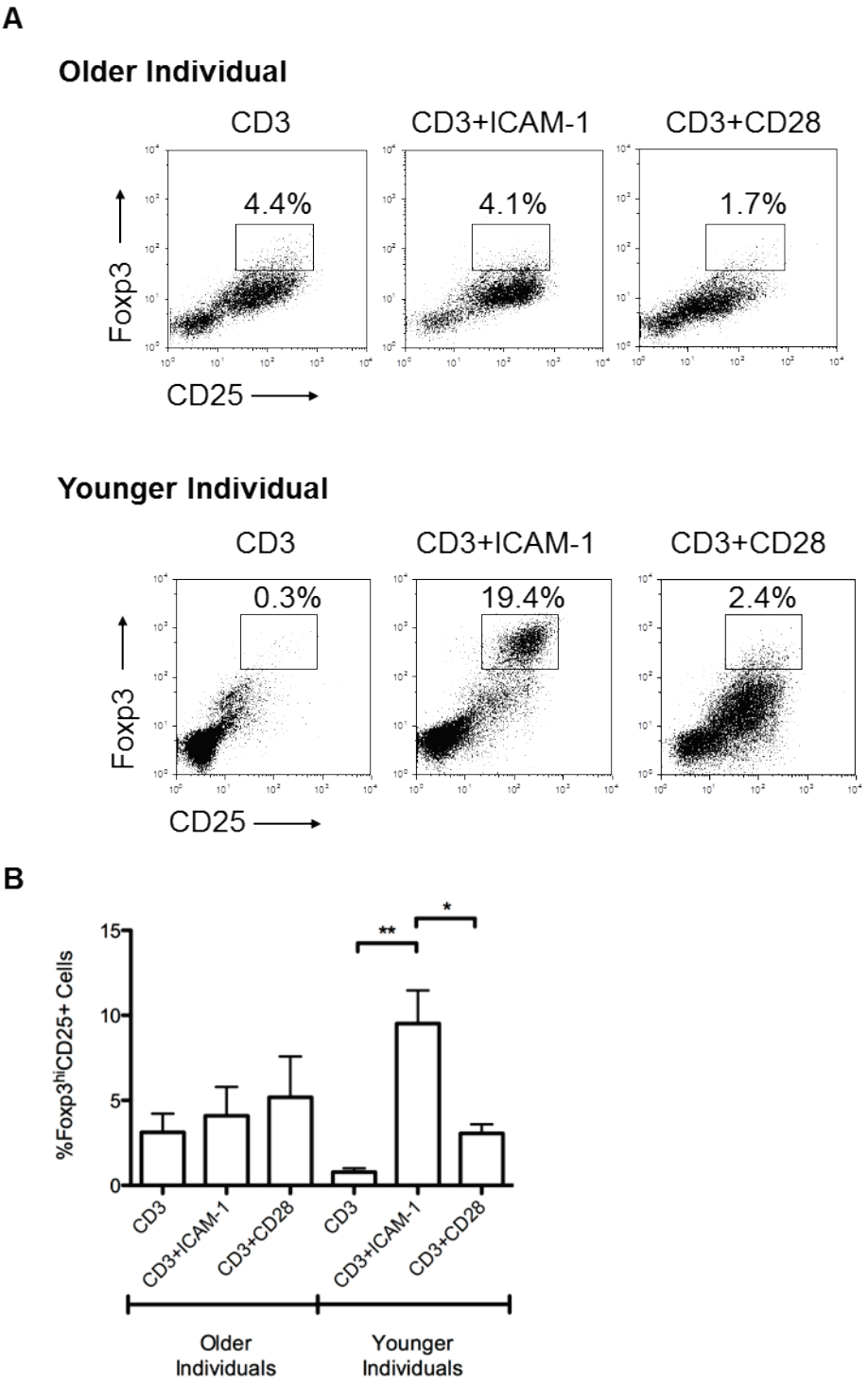
A
Older Individual
CD3
CD3+ICAM-1
CD3+CD28
4.4%
4.1%
1.7%
Foxp3
CD25
Younger Individual
CD3
CD3+ICAM-1
CD3+CD28
0.3%
19.4%
2.4%
Foxp3
CD25
B
%Foxp3hiCD25+ Cells
15
10
5
0
**
*
CD3
CD3+ICAM-1
CD3+CD28
CD3
CD3+ICAM-1
CD3+CD28
Older Individuals
Younger Individuals

Figure 3.6. Naïve CD4+ T cells from an older individual could differentiate to cells with a Foxp3^{hi} CD25+ T_{reg} phenotype. Exogenous TGF-β1 (10 ng/mL) plus IL-2 (10 U/mL) were added to cultures at the beginning of stimulation. Naïve CD4+ T cells were stimulated in the presence of exogenous TGF-β1 plus IL-2 for 7 days. Representative of 1 experiment using cells from an older subject.

Figure 3.6

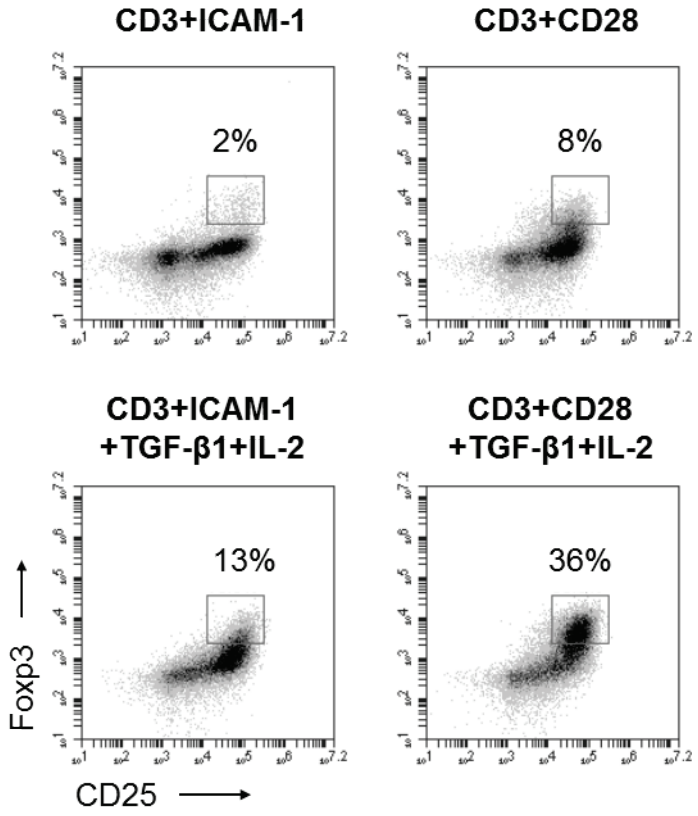
CD3+ICAM-1
CD3+CD28
2%
8%
CD3+ICAM-1
+TGF-β1+IL-2
CD3+CD28
+TGF-β1+IL-2
13%
36%
Foxp3
CD25

Figure 3.7. Naïve CD4+ T cells from older individuals secrete TGF-β1 after costimulation through either ICAM-1 or CD28. Naïve CD4+ T cells from older individuals were stimulated as indicated for 7 days. Cell culture supernates were collected, clarified by centrifugation, and analyzed by ELISA. Data are the means of duplicate samples from 2 experiments using cells from older individuals and 4 experiments using cells from younger individuals ± SEM. Differences between older and younger individuals, comparing the same stimulation treatments, were not statistically significant. TGF-β1 secretion data for younger individuals can also be found in Figure 2.4B.

Figure 3.7

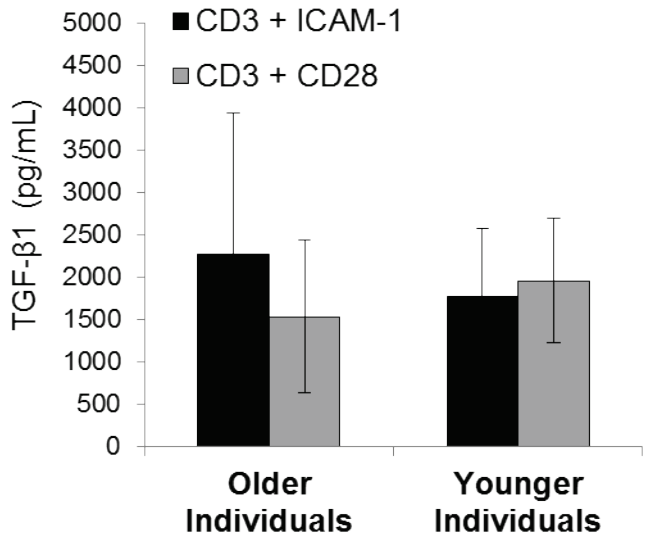
CD3 + ICAM-1
CD3 + CD28
TGF-β1 (pg/mL)
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Older Individuals
Younger Individuals

Table 3.1. Summary of results in our differentiation studies using naïve CD4+ T cells from older and younger individuals. T_{reg} differentiation results are presented in Chapter 2 of this dissertation and Williams *et al.* (23) (younger individuals) and Chapter 3 of this dissertation (older individuals). Results for effector and memory differentiation were presented in Dr. Jake Kohlmeier's dissertation (26) and Kohlmeier *et al.* (22) (younger individuals) and are presented in Abby Dotson's dissertation (older individuals).

Table 3.1

	Older Individuals		Younger Individuals	
	ICAM-1	CD28	ICAM-1	CD28
Differentiation to Effector Phenotype	+	+	+	+
Differentiation to Memory Phenotype	+	+	+	+
Differentiation to T_{reg} Phenotype	**-**	**-**	**+**	**-**

DISCUSSION

Accumulating data indicate that T cell activation and differentiation are modified as we age. In this chapter, we studied the role of the costimulatory molecules ICAM-1 and CD28 on the process of naïve CD4+ T cell differentiation *in vitro*. I studied differentiation to cells with a T_{reg} phenotype while Abby Dotson studied differentiation to cells with effector and memory phenotypes. Our data suggest that some outcomes of costimulation may differ between naïve CD4+ T cells from older and younger individuals, while others are the same.

When we compared the percentage of Foxp3+ cells in the total CD4+ population in PBMCs, we detected similar percentages between older and younger subjects. The mean percentage of Foxp3+ cells was slightly higher for older individuals, but the increase was not statistically significant (**Fig. 3.1**). Our data are comparable to those of Hwang *et al*. (16). However, these data contrast with other studies showing increases in the percentage of T_{reg} cells with age (11, 14).

While thymic output of naïve T cells is known to decrease with aging, less is known about possible phenotypic and functional changes in naïve T cells in older individuals. Naïve CD4+ T cells from older and younger subjects had similar phenotypes (**Fig. 3.2, 3.3**). A limitation to these results is that some experiments were preliminary and only included 1 sample per age group. Additional experiments should be performed to support these data and to study the expression of other proteins of interest.

We employed our *in vitro* naïve T cell differentiation system to determine if there were differences in outcome after costimulation of naïve CD4+ T cells from older individuals compared with younger individuals. Cells from older individuals expanded in culture the best after costimulation through ICAM-1 (data not shown, data are presented in Abby Dotson's

dissertation). Abby Dotson also showed that naïve CD4+ T cells from older individuals could differentiate to effector and memory phenotypes after costimulation through either ICAM-1 or CD28 (**Fig. 3.4**). With the exception of 2 older subjects, costimulation of naïve CD4+ T cells did not generate a population of cells with a Foxp3^{hi} T_{reg} phenotype (**Fig. 3.5A,B**). This contrasts with our previous results studying naïve CD4+ T cells from younger individuals in which costimulation through ICAM-1 routinely yielded a T_{reg} population.

To determine if the reduced differentiation to cells with a Foxp3^{hi} T_{reg} phenotype was due to an ICAM-1-specific effect or a general defect in T_{reg} differentiation, we analyzed whether T_{reg} differentiation could occur in the presence of exogenous cytokines. Cells with a Foxp3^{hi} T_{reg} phenotype were induced when exogenous TGF-β1 plus IL-2 were added to stimulated cultures in one experiment (**Fig. 3.6**). These preliminary data suggest that naïve CD4+ T cells from older individuals had the ability to differentiate to T_{reg} cells since they differentiated using stimuli that are generally known to generate T_{reg} cells. However, it should be noted that this experiment was only performed once. These preliminary results show that the diminished T_{reg} induction seen using naïve cells from older individuals can be overcome with the addition of exogenous TGF-β1 plus IL-2.

We do not yet have definitive answers for why 1) naïve CD4+ T cells from older subjects did not typically differentiate to Foxp3^{hi} T_{reg} cells after costimulation through ICAM-1, or 2) in 2 experiments, the older subjects had cells that differentiated to a Foxp3^{hi} T_{reg} population, while in the other 7 experiments, T_{reg} cells did not differentiate. Decreased T_{reg} induction could possibly occur if TCR or ICAM-1 signaling strength differed between naïve CD4+ T cells from older and younger individuals. However, costimulation through ICAM-1 up-regulated expression of CD25 (IL-2Rα), which is a protein found on both T_{reg} cells and activated T cells, indicating that cell

activation occurred (**Fig. 3.5**). As further evidence that T cell activation occurred after ICAM-1 costimulation, we observed expansion of cell number (data not shown, are presented in Abby Dotson's dissertation) and differentiation to effector and memory cells (**Fig. 3.4**). Costimulation through ICAM-1 also led to the production of TGF-β1 in concentrations similar to that produced by naïve CD4+ T cells from younger individuals. Therefore, it appears that signal strength through the TCR plus ICAM-1 was sufficient.

Another possibility is that a specific signaling pathway was impaired in cells from older individuals. One possibility might be lower IL-2 production. Secretion of IL-2 by T cells has been shown to be reduced in both older individuals (27) and aged mice (28). Haynes *et al.* further showed that when exogenous IL-2 was added to naïve CD4+ T cells from aged mice, effector cell differentiation and production of IL-2 was increased to levels seen in young mice (28). We and others have previously shown that IL-2 was necessary for T_{reg} differentiation in cells from younger individuals (Fig. 2.6) (23). We did not collect data on IL-2 secretion from cells from older individuals, so we do not know if there is a difference in IL-2 production between age groups. If less IL-2 was secreted by stimulated naïve CD4+ T cells from older individuals, this might explain why we see diminished T_{reg} induction from older individuals in our study.

Another possibility is that T_{reg} cells can differentiate after ICAM-1 costimulation, but the timing of differentiation is different. We did experiments from Days 3-14 to detect T_{reg} cells, but it was rare that there were enough starting naïve CD4+ T cells available to do a time-course experiment for each individual. Therefore, most often the cells from older subjects were tested on Day 7 of stimulation. So, it remains possible that we missed the time-point that they differentiated. In addition, even though we were most interested in detecting $Foxp3^{hi}$ T_{reg} cells, it

is possible that there were Foxp3^{int} T_{reg} cells that differentiated. Performing suppression assays using magnetically isolated CD25+ cells would reveal whether there were suppressor cells in the stimulated cultures even though Foxp3^{hi} cells were not present.

Naïve CD4+ T cells did differentiate to Foxp3^{hi} cells in 1 experiment after costimulation through ICAM-1 or CD28 and in a second experiment after costimulation through CD28. The heterogeneity in results might suggest that some older individuals have cells that behave more like cells from younger individuals than others. The subject who displayed Foxp3^{hi} cells after costimulation through ICAM-1 and after costimulation through CD28 was one of the youngest of the older individuals tested. The other subject who displayed Foxp3^{hi} cells after costimulation through CD28 had been tested in other experiments and had not shown induction of Foxp3^{hi} cells in the past. It remains possible that these two results are anomalous due to some artifact in the culture that led to differentiation occurring in these two individuals. Also, because we do not gather data regarding their medical histories, we do not know if any of the subjects have underlying health issues that might possibly skew the immune response toward or away from T_{reg} differentiation. Also, genetic variability and differences in previous exposure to infectious agents and other environmental factors would be expected to lead to heterogeneity among immune responses.

The studies described in this Chapter and in Abby Dotson's dissertation describe a role for ICAM-1 in the activation and differentiation of naïve CD4+ T cells obtained from older individuals. Even though aging has been reported to affect activation and differentiation, naïve CD4+ T cells from older individuals can still respond to costimulation through ICAM-1 *in vitro* to cause expansion in cell number and differentiation to effector and memory subsets. However, differentiation to T_{reg} cells seemed to be impaired in most cases. If one were to greatly speculate

and translate our *in vitro* results to an *in vivo* immune response in a microenvironment in which the naïve CD4+ T cell were stimulated by an APC expressing an ICAM-1 ligand in the absence of T_{reg}-inducing cytokines, one might predict that naïve CD4+ T cells from older individuals might favor differentiation to effector and memory, but not T_{reg} cells. This differentiation profile would be expected to promote defense against infections and cancers, but might also promote autoimmunity and inflammation.

As mentioned in the Introduction, there are some differences between data presented in this Chapter and similar studies by other investigators, as well as inconsistencies within the immunosenescence literature in general. Differences in mouse studies could arise from strain differences between C57Bl/6 and Balb/c mice. Not only do these mouse strains favor different T_h responses, Balb/c mice have higher percentages of CD4+CD25^{hi} T_{reg} cells (29). In human studies, selecting different criteria based on age, sex, and health of the participants could affect results. For example, one study found that changes in cytokine production that occurred during aging varied between the groups of women and men (30). In our study, older individuals were 65 years or older, were all men, and were in at least reasonably good health (*i.e.* no acute severe illness, not taking immunosuppressive drugs). Some possibly beneficial changes to an expanded study would be to enroll subjects from a variety of ethnic groups and socioeconomic backgrounds and both sexes, which would better represent the aging population in the U.S. Overall, aging research will be improved by collaborative efforts and long-term longitudinal studies.

Studying costimulatory molecules during aging will help us understand how the immune system is remodeled during aging and how this affects disease risk. Studies have indicated that improving costimulatory signaling can improve immune responses. For example, a study in

Balb/c mice showed that aged mice did not reject a tumor well unless the tumor expressed the costimulatory ligand CD80 or agonist anti-OX40 antibody was added (31). The costimulatory molecule CD28 is decreased on many T cells in older individuals, especially CD8+ T cells, but also CD4+ T cells (32). However, the costimulatory molecule LFA-1 is increased on T cells in aging and has been proposed to perhaps contribute to autoimmunity (21). Much is left to be learned about the contributions of costimulatory molecules to effective immune responses in older individuals. From a mechanistic standpoint, it will be important to determine what signaling pathways ICAM-1 uses and how these might be altered in older individuals leading to possible T_{reg} differentiation differences. From a therapeutic standpoint, it might be useful to stimulate through ICAM-1 to strengthen immune responses. This might be especially useful if follow-up studies were to confirm that costimulation through ICAM-1 amplified an effector and memory response, but not a T_{reg} response in most older individuals.

CHAPTER 3 ACKNOWLEDGEMENTS

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CHAPTER 4

FUNCTION OF ICAM-1 ON MOUSE T CELL ACTIVATION AND DIFFERENTIATION

ABSTRACT

Differentiation of naïve T cells to populations of effector, memory, and regulatory T (T_{reg}) cells occurs after signaling through the T cell receptor, accompanied by signals from costimulatory molecules and cytokines in the cellular microenvironment. Our lab has shown that ICAM-1 expressed on **human** T cells can receive a costimulatory signal, leading to activation and differentiation to effector, memory, and T_{reg} subsets. In this chapter, we investigated whether ICAM-1 could similarly deliver a costimulatory signal that would influence cellular differentiation in **mouse** T cells. We used an *in vitro* culture system and commercially available antibodies to attempt to signal through ICAM-1. However, we did not observe differentiation of mouse CD4+ T cells to a T_{reg} phenotype after costimulation through ICAM-1 unless exogenous cytokines were added to the cultures. Induction of mouse T_{reg} cells after ICAM-1 costimulation plus cytokine signaling was not greater than that observed with controls, indicating that ICAM-1 was not involved in the process. These data may suggest possible differences in the process of T_{reg} induction in human T cells compared with mouse T cells. In a related project, we studied the role of ICAM-1 on T cell activation and differentiation *in vivo* in mice deficient in ICAM-1 or deficient in both ICAM-1 and CD28. Mice were infected with vesicular stomatitis virus (VSV) and the ability of CD8+ T cells to become activated and secrete IFN-γ, display cytolytic function, and differentiate to memory cells was tested. Our results indicated that mice deficient in both ICAM-1 and CD28 could still initiate cell-mediated immune responses, although sometimes at different levels than observed in wild-type mice.

NOTES

Some data presented in this chapter are included in a paper in preparation (Williams *et al*.).

INTRODUCTION

Our group has previously demonstrated *in vitro* that ICAM-1 resident on the human T cell surface can also act as a costimulatory molecule on the T cell itself by activating phosphatidylinositol 3-kinase, increasing proliferation, and inducing cytokine secretion (1) and can guide the *in vitro* differentiation of human naïve CD4+ T cells to both Foxp3+ T_{reg} cells (2) and to CD45RA(-)CD11a+CD27(-) memory cells (3). Other groups have studied the function of ICAM-1 *in vivo* in mouse models of disease. One mouse strain that is deficient in ICAM-1, *Icam1*^{tm1Jcgr}, was made by disrupting exon 4 in the *Icam1* gene (4). This ICAM-1 deficient mouse showed increased numbers of circulating neutrophils and lymphocytes, decreased MLR and DTH responses, and was resistant to the lethal effects of LPS or *S. aureus* exotoxin (4). Another ICAM-1 deficient mouse strain is the *Icam1*^{tm1Bay} which was made by disrupting exon 5 in the *Icam1* gene (5). However, there is evidence that these two strains of ICAM-1 deficient mice are not truly ICAM-1 null. While they do not express full-length ICAM-1 which contains 5 extracellular domains, the ICAM-1 "deficient" mice can express alternatively spliced ICAM-1 isoforms. Some of these isoforms still contain the LFA-1 binding site, and in the *Icam1*^{tm1Bay} mouse some of the isoforms contain the Mac-1 binding site. True ICAM-1 null mice were later generated by deleting the entire *Icam1* coding region (6).

The *Icam1*^{tm1Jcgr} strain is the ICAM-1deficient strain most commonly used, and will be referred to as ICAM-1$^{-/-}$ in this chapter unless otherwise indicated. Interestingly, ICAM-1$^{-/-}$ mice have fewer T_{reg} cells and a stronger response to tuberculosis infection (7). In the experimental autoimmune encephalomyelitis (EAE) mouse model for MS, disease progression is dependent upon the strain of ICAM-1 mutant mouse studied, which the authors concluded was related to the

alternatively-spliced ICAM-1 isoforms expressed. The *Icam1*^{tm1Jcgr} strain actually had attenuated EAE symptoms compared to wild-type mice and to *Icam1*^{tm1Bay} mice (8).

There are some notable biochemical differences between human and mouse ICAM-1. Mouse and human ICAM-1 share only 65% DNA homology and 50% protein homology (9). However, the ICAM-1 residues that are important for LFA-1 binding are conserved between the two species (10). The human ICAM-1 cytoplasmic domain contains a putative SH3 domain-interacting PxxP motif (11) and an IKKYRLQ sequence with some similarity to SH2 domain-interacting immunoreceptor tyrosine inhibition motifs (ITIMs) (12). The PxxP sequence is not present in mouse ICAM-1, and an IRIYKLQ sequence is found in mouse instead of the human ICAM-1 IKKYRLQ sequence.

In Part I of this chapter, we studied whether signaling through mouse ICAM-1 resident on the mouse T cell surface would lead to differentiation. We observed that, in contrast to our results with human T cells, ICAM-1 costimulation did not lead to differentiation to mouse inducible T_{reg} cells. In Part II of this chapter, we studied whether ICAM-1 and CD28 are required for CD8+ T cell activation and differentiation in a mouse model of VSV infection. We observed that in the absence of both ICAM-1 and CD28, CD8+ T cells could still become activated and differentiate to both CTLs and memory CD8+ T cells, although some differences between groups were observed.

MATERIALS AND METHODS

Part I

Mice

C57Bl/6J and Balb/cJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Protocols were approved by the University of Kansas Institutional Animal Care and Use Committee.

Cell Purification

Total splenocytes were isolated from C57Bl/6 mice of typically age 7 to 16 weeks as described in Protocol 8 in the Appendix to this Dissertation. Splenocytes were purified by gently mincing over a strainer and then lysing red blood cells using ACK Lysing Buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 μM Na_2EDTA, pH = 7.2-7.4). CD4+ T cells were purified using StemSep Mouse CD4+ T Cell Enrichment Kits and Total T cells were purified using StemSep Mouse T Cell Enrichment Kits (StemCell Technologies) using the manufacturer's recommended protocols. Cells were cultured in complete RPMI 1640 medium (Mediatech, Herndon, VA), which contained 10% FBS (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen/Life Technologies, Carlsbad, CA). In addition, 1% sodium pyruvate, 1% non-essential amino acids (Invitrogen/Life Technologies), and 50 μM β-mercaptoethanol (Calbiochem) were added to the complete RPMI 1640 medium in many of the mouse cell culture experiments.

Antibodies and Reagents

Antibodies used to attempt to stimulate mouse cells are listed in **Table 4.1**. The following clones of anti-CD3 were used: OKT3 (eBioscience or hybridoma purchased from ATCC), 145-2C11 (BD Biosciences, San Jose, CA), KT3 (Chemicon), 500A2 (BD Biosciences), and C363.29B (Southern Biotech). The following clones of anti-ICAM-1 were used: R6.5D6 (BioXCell or hybridoma purchased from ATCC), 3E2 (BD Biosciences), 166623 (R&D Systems, Minneapolis, MN), YN1/1.7.4 (eBioscience), BE2961 (Biodesign), 3E2B (Chemicon), and functional grade or purified KAT-1 (eBioscience). Anti-CD28 (clone 37.51) was purchased from BD Biosciences. All stimulating antibodies tested were anti-mouse antibodies with the exceptions of OKT3 and R6.5D6 which are anti-human antibodies. Flow cytometry staining of mouse T cells was performed using anti-Foxp3-PE (eBioscience) and anti-CD25-TriColor (Invitrogen/Life Technologies). Recombinant human TGF-β1 was purchased from R&D Systems and used at 2 ng/mL, and recombinant human IL-2 was purchased from Boehringer Mannheim/Roche (Mannheim, Germany) and used at 50 U/mL. Although these cytokines are derived from human cytokine sequences, they are known to cross-react with mouse. CFSE (5-(and-6)-carboxyfluoresceindiacetate, succinimidyl ester) was purchased from Molecular Probes/Life Technologies. Flow cytometry was performed using a FACScan (BD, San Jose, CA) or an Accuri C6 (Accuri Cytometers, Ann Arbor, MI), and data was analyzed using CellQuest (BD), CFlow (Accuri) and FlowJo software (Tree Star, Inc., Ashland, OR).

T Cell Stimulation

Stimulation of mouse CD4+ T cells or total T cells was performed using plate-bound antibodies as described in the Chapter 2 Materials and Methods. Antibodies in PBS were adhered to tissue-culture treated flat-bottom 96-well plates by incubation at 37 degrees for 2 hours, followed by

washing of the wells 3 times with PBS. For experiments using mouse cells, antibodies were tested at the concentrations listed in **Table 4.1**. Most mouse T_{reg} experiments used anti-CD3ε clone 500A2 at 0.5 μg/mL, anti-ICAM-1 clone KAT-1 or clone YN1/1.7.4 at 10 μg/mL, and anti-CD28 clone 37.51 at 2.5 μg/mL. Cells were stimulated at $1.5x10^6$ cells/mL in 200 μL of culture medium.

Suppression Assay

Mouse CD4+ T cells were stimulated for 5 days using the parameters described in this chapter. On day 5, the cells were spun over Lympholyte-M (Cedarlane Laboratories, Burlington, NC) to remove debris as described in the Appendix in Protocol 9. These stimulated cells served as potential suppressor cells in the experiment. Also on day 5, new total T cells were purified from mouse spleen, and then labeled with CFSE dye. These total T cells served as responder T cells. Cells were mixed in culture at a 1:2 Stimulated to Responder cell ratio. Co-cultured cells were stimulated with anti-CD3 plus anti-CD28 for between 3-5 days. Proliferation of the CFSE-labeled responder cells was measured by gating out the unlabeled stimulated cells and determining proliferation of the responder cells only. Further details are provided in the Appendix in Protocol 10.

Part II

Mice

C57Bl/6J mice, ICAM-$1^{-/-}$ mice (strain B6.129S4-*Icam1*^{tm1Jcgr}/J), $CD28^{-/-}$ mice (strain B6.129S2-*Cd28*^{tm1Mak}*/J*), and TCR$\beta^{-/-}$ mice (strain B6.129P2-*Tcrb*/J) were purchased from The Jackson Laboratory. ICAM-$1^{-/-}$ and $CD28^{-/-}$ mice were crossed to generate ICAM-$1^{-/-}CD28^{-/-}$ mice, and

genotyping was performed by Dr. Jake Kohlmeier as described in his Dissertation (13). Protocols were approved by the University of Kansas Institutional Animal Care and Use Committee.

Reagents

MHC Class I specific VSV peptide VSV NP_{52-59} (sequence RGYVYQGL) was obtained from SynPep (Dublin, CA). Flow cytometry staining of mouse T cells was performed using anti-IFN-γ-FITC (BD Biosciences), anti-F4-80-PE (Caltag/Life Technologies) and either anti-CD8a-PerCP (BD Biosciences) or anti-CD8a-TriColor (Caltag/Life Technologies). Recombinant human IL-2 was purchased from Boehringer Mannheim/Roche and used at 50 U/mL. Flow cytometry was performed using a FACScan, and data was analyzed using CellQuest (BD) and FlowJo software (Tree Star, Inc.).

Vesicular Stomatitis Virus Infection

Vesicular Stomatitis Virus (VSV) Indiana strain was purchased from ATCC. Dr. Jake Kohlmeier grew the virus in cell culture and determined the virus titer. Mice were infected *i.v.* with $1x10^6$ PFU VSV via tail vein injection. This is a nonlethal VSV dose in immunocompetent mice. Mice were typically infected at 8-10 weeks of age. Immune responses were tested at day 7 post-infection for acute responses or day 70 post-infection for memory responses.

Cell Purification

Total splenocytes were isolated from mice as described in Protocol 8 in the Appendix to this Dissertation. Splenocytes were purified by gently mincing over a strainer and then lysing red

blood cells using ACK Lysing Buffer. Total T cells were purified using StemSep Mouse T Cell Enrichment Kits (StemCell Technologies). The cell culture medium used was RPMI 1640 (Mediatech), which contained 10% FBS (Atlanta Biologicals), 2 mM L-glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (Invitrogen).

T cell Adoptive Transfer

Total T cells were isolated from splenocytes as described above. 5 x 10^6 donor T cells in sterile PBS were injected via tail vein into 4-6 week old recipient TCR$\beta^{-/-}$ mice. 4 weeks later, recipient mice (at 8-10 weeks of age) were infected with 1x10^6 PFU of VSV via tail vein injection.

Intracellular Cytokine Assay

IFN-γ production by CD8+ T cells was determined using an intracellular cytokine assay as described in Appendix Protocol 11. Splenocytes were purified from mice on day 7 post infection. Splenocytes were incubated in round-bottom 96-well plates for 5-6 hours at 1x10^6 cells/well in 200 μL complete RPMI1640 medium containing 3 μM monensin, 50 U/mL IL-2, with or without 2 μg/mL VSV peptide. After incubation, splenocytes were surface stained for CD8 and F4/80 and intracellularly stained for IFN-γ as described in Appendix Protocol 11. Permeabilization buffers and monensin were purchased from BD Biosciences.

In vivo cytotoxicity assay

The cytolytic ability of CD8+ T cells was determined using *in vivo* cytotoxicity assays as described in Appendix Protocol 12. Splenocytes were purified from uninfected donor mice and stained with 5 μM PKH26 dye (Sigma, St. Louis, MO) following the manufacturer's protocol.

The stained cells were separated into two groups: a $CFSE^{lo}$ group that was stained with 0.5 μM CFSE and a $CFSE^{hi}$ group that was stained with 5 μM CFSE. The $CFSE^{hi}$ group was incubated with 5 μg/mL VSV peptide for 1 hour. The $CFSE^{lo}$ (unpulsed targets) and $CFSE^{hi}$ (VSV peptide-pulsed targets) groups were combined. $5x10^6$ unpulsed targets and $5x10^6$ VSV-pulsed targets were *i.v.* injected into recipient mice 7 days post-infection. 16 hours later, splenocytes were purified from recipient mice and the Percent Specific Lysis was determined using the equation used by Byers *et al.* (14). The Percent Specific Lysis was calculated as: [(# of unpulsed targets x *A* - # of VSV peptide-pulsed targets)/# of unpulsed targets x *A*] x 100%, where *A* = [number of unpulsed targets/# of VSV peptide-pulsed targets] in uninfected control mice.

Statistical Analysis

One-way ANOVA and Tukey's post-test were performed to compare the results from each mouse genotype using GraphPad Prism (15). GraphPad Prism was used to detect any possible outliers using the Robust regression and OUTlier removal (ROUT) method for automatic outlier elimination and the Grubb's test. No outliers were identified using either method.

RESULTS

Part I: Costimulation of mouse T cells through ICAM-1 may differ from costimulation of human T cells

Various costimulatory molecules can participate in the activation and differentiation of naïve T cells, and our previous results have revealed a role for ICAM-1 in the process of differentiation to effector, memory and T_{reg} subsets from human naïve CD4+ T cells.

To follow our studies in human T cells, we next attempted to study the effects of ICAM-1 costimulation on the differentiation of mouse CD4+ T cells. Strains of mice used for experiments were C57Bl/6 and Balb/c. We chose to begin experiments using total CD4+ T cells, rather than naïve CD4+ T cells, since we did not have a satisfactory method of obtaining mouse naïve CD4+ T cells. The starting CD4+ T cell population purified from spleen is expected to contain approximately 70% $CD44^{lo}$ naïve cells (16). To determine if ICAM-1 could generate a costimulatory signal to initiate C57Bl/6 T cell activation and differentiation, we used anti-CD3 plus anti-ICAM-1 antibodies to attempt to costimulate mouse total T cells. As shown in **Figure 4.1**, adding anti-CD28 antibody caused a dramatic increase in proliferation, whereas adding anti-ICAM-1 either caused no effect or seemed to have a blocking effect. It should be noted that this experiment was only performed once using total T cells; however, similar results were obtained in two additional experiments using total CD4+ T cells (data not shown). These results differ from those previously published by another group, in which mouse CD4+ and CD8+ T cells expanded in number when anti-ICAM-1antibody was added to the stimulation regimen, although the stimulation conditions varied between our experiments and their experiments (17).

Although cellular proliferation did not appear to be increased after ICAM-1 costimulation in our experiments, it is possible that cellular differentiation was affected by ICAM-1

costimulation. A collaborator on this project, graduate student Abby Dotson, studied the capacity of mouse Balb/c CD4+ T cells to differentiate to central memory cells following stimulation through ICAM-1 in one preliminary experiment. She found that after gating on the $CD44^{hi}$ cells, more of the $CD44^{hi}$ cells had a CD127+CD62L+ central memory phenotype after costimulation through either ICAM-1 or CD28 when compared to stimulation through CD3 alone (data not shown, data appear in Abby Dotson's dissertation). This preliminary experiment suggested that differentiation might be altered after addition of anti-ICAM-1 antibodies to the culture conditions.

To determine whether ICAM-1 costimulation could induce the differentiation of mouse T_{reg} cells, C57Bl/6 splenic CD4+ T cells were stimulated through CD3+ICAM-1 for 5 days using plate-bound antibodies and then analyzed for the T_{reg} phenotypic markers Foxp3 and CD25. Several clones and concentrations of anti-CD3 and anti-ICAM-1 antibodies were tested through the course of this project (**Table 4.1**). As shown in **Figure 4.2A**, costimulation through ICAM-1 on mouse CD4+ T cell did not yield Foxp3+ cells, nor did the control stimulations. However, when the exogenous cytokines TGF-β1 and IL-2 were added to stimulated cultures, a sizable Foxp3+ population was observed (**Fig. 4.2B**). This was most likely due to the effects of TGF-β1 and IL-2 rather than an ICAM-1 specific effect, since this Foxp3+ population was observed with CD3 stimulation alone and with CD28 costimulation. The contribution of TGF-β1 to T_{reg} induction has previously been reported by others (18, 19).

Even though costimulation of mouse CD4+ T cells through ICAM-1 did not generate a population with a T_{reg} phenotype, we questioned whether ICAM-1 costimulation could still lead to cells with suppressive capabilities. We performed a suppression assay similar to that used by other investigators to establish T_{reg} function (20). In our assay, we used CFSE-labeled total

mouse T cells as responder cells and mouse CD4+ T cells previously stimulated through CD3+ICAM-1 as potential suppressor cells at a 1:2 Stimulated:Responder cell ratio (**Fig. 4.3**). Responder cell proliferation did not decrease when cells previously stimulated through CD3+ICAM-1 were added to cultures. It should be noted that in some experiments, the ratio of Stimulated:Responder cells was less than 1:2 due to a low number of viable stimulated cells recovered after the Lympholyte-M spin. In some instances, responder cell proliferation diminished very slightly when cells stimulated in the presence of TGF-β1 plus IL-2 were added as potential suppressor cells, but this was not a consistent effect. These data demonstrate that mouse CD4+ T cells stimulated through CD3+ICAM-1 using commercially available antibodies do not differentiate into T_{reg} cells. These results contrast markedly with our studies in which T_{reg} induction occurs after costimulation through ICAM-1 using human naïve CD4+ T cells obtained from younger individuals.

Part II: Neither ICAM-1 nor CD28 is required for CD8+ T cell activation and differentiation during VSV infection

In a separate but related project, the role of ICAM-1 and CD28 in T cell activation and differentiation were studied using mice deficient in these costimulatory molecules. Former graduate student, Dr. Jake Kohlmeier, crossed the B6.129S4-*Icam1*^{tm1Jcgr} and B6.129S2-*Cd28*^{tm1Mak} mice to generate mice deficient in both ICAM-1 and CD28. ICAM-1$^{-/-}$CD28$^{-/-}$ mice were viable and fertile and had normal percentages of peripheral CD4+ and CD8+ T cells in the spleen. Jake found that ICAM-1$^{-/-}$ mice, CD28$^{-/-}$ mice, and ICAM-1$^{-/-}$CD28$^{-/-}$ mice each had subtle differences in thymic double-negative stages of development compared to wild-type C57Bl/6 mice (13). Jake began experiments to test the immune response of ICAM-1$^{-/-}$CD28$^{-/-}$

mice during VSV infection, and I later completed the project. The data presented in this Chapter are an accumulation of the VSV infection experiments that we completed.

We directed our study to CD8+ T cells which play a principal role in cell-mediated immune responses. During viral infection, naïve CD8+ T cells become primed in peripheral lymphoid organs (*e.g.* lymph nodes, spleen) by APCs presenting viral antigen on MHC Class I molecules. Activated CD8+ T cells undergo clonal expansion, differentiate into cytotoxic T lymphocytes (CTLs), and acquire the ability to migrate to the site of infection. CTLs will act at the site of infection by secreting cytokines such as IFN-γ and killing virus-infected cells through perforin and granzymes. Effector CTLs will die after the acute infection has been cleared, but a subset of memory cells will remain that can be activated upon a secondary infection with the same virus (21). During VSV infection, components of both the cell-mediated and humoral immune responses are activated. Studies using mice deficient in either T cells or B cells have shown that antibodies are required in the early stage of VSV infection, at least if the route of infection is *i.v.* (22). T cells are required in later stages of the infection (23).

Cd28^{tm1Mak} mice that are deficient in CD28 have T cells with a decreased response to lectin stimulation, decreased basal antibody concentrations, impairments in antibody class switching, and lack germinal center formation after VSV infection (24, 25). CD28-deficient mice have previously been shown to have reduced, but not absent, IFN-γ production by CD8+ T cells after VSV infection (26). Studies using mice deficient in both B7-1 and B7-2 have also shown impairments in antibody class switching and CTL generation (27). CD8+ T cells are more dependent on CD28 costimulation during VSV infection than during LCMV infection, due to a lower extent and duration of infection in VSV compared to LCMV (28).

Icam1^{tm1Jcgr} mice have increased leukocyte counts in the blood, decreased MLR and DTH responses, and show increased survival after injection of LPS or *S. aureus* exotoxin compared to wild-type mice (4). ICAM-1 deficient mice have decreased T cell activation and proliferation *in vitro* and *in vivo* due to a lack of ICAM-1 on the APC (29). ICAM-1 expression on DCs is critical for stable interactions with CD8+ T cells to generate T cell memory (30). *In vitro* experiments using leukocytes from CD28 deficient mice have demonstrated that stimulation provided by ICAM-1 on the APC is required if CD28 is absent on the T cell (31). ICAM-1 on the APC is especially important in providing a second signal to primed CD8+ T cells since many target cells do not express B7-1 or B7-2. Together, these studies suggest that if both CD28 and ICAM-1 were missing, the immune response to a viral infection would be expected to be impaired.

To assess CD8+ T cell function with and without the contribution of ICAM-1 and CD28, we infected C57Bl/6 mice, ICAM-1$^{-/-}$ mice, and ICAM-1$^{-/-}$CD28$^{-/-}$ mice with 1x10^6 PFU of VSV strain Indiana by *i.v.* tail vein injection. After 7 days of infection, splenocytes were purified from infected mice and *in vitro* cytokine assays were performed to determine whether the lack of two important costimulatory molecules would affect activation of CD8+ T cells during viral infection. As expected for uninfected control C57Bl/6 mice, very few CD8+ T cells were specific for VSV antigen, and therefore, very few CD8+ T cells produced IFN-γ (**Fig. 4.4A**, left panels). Very few CD8+ T cells produced IFN-γ when VSV peptide was not added to the culture (**Fig. 4.4A**, bottom panels). The cells producing IFN-γ in the groups with no peptide may represent bystander activation of CD8+ T cells, or represent a low level of nonspecific flow cytometry staining. In all three genotypes of mice (C57Bl/6, ICAM-1$^{-/-}$, ICAM-1$^{-/-}$CD28$^{-/-}$), VSV peptide addition to the culture led to a greatly increased percentage of CD8+ T cells

producing intracellular IFN-γ. This indicated that there were virus-specific CD8+ T cells that had become activated and clonally expanded during the 7 days of infection *in vivo*. Interestingly, slightly increased percentage of cells were producing IFN-γ in the ICAM-$1^{-/-}$ mice, while a reduced percentage of cells were producing IFN-γ in the ICAM-$1^{-/-}$CD$28^{-/-}$ mice (**Fig. 4.4B**). Unfortunately, CD$28^{-/-}$ mice were not available for this study as a comparison group.

Because ICAM-1 is expressed on many different cell types, including APCs, we wanted to narrow our study to the effect of ICAM-1 costimulation on the T cell alone. To accomplish this, we performed T cell adoptive therapy experiments whereby we purified total T cells from each of the genotypes of interest and *i.v.* injected them into TCR$\beta^{-/-}$ mice which lack αβ T cells. Four weeks after T cell adoptive transfer, the mice were infected with VSV. IFN-γ production by CD8+ T cells was analyzed 7 days post-infection (**Fig. 4.5A, B**). Although differences were not statistically significant, results followed the same trends as the acute response shown in **Figure 4.4**, with a slightly heightened response in mice with transferred ICAM-$1^{-/-}$ T cells and a lowered response in mice with transferred ICAM-$1^{-/-}$CD$28^{-/-}$ T cells. It is of note that the TCR$\beta^{-/-}$ mice without transferred T cells still survived VSV infection. Others have shown that mice lacking αβ T cells still have functional γδ T cells that can produce IFN-γ and provide help for antibody class switching (32).

Next, an *in vivo* cytotoxicity assay was performed to determine if CD8+ T cells had differentiated into functional CTLs with cytolytic function. Similar assays have been previously performed by other groups (14, 33). Total splenocytes were purified from control uninfected mice and stained with PKH26 dye. The splenocytes were divided in two groups as described in the Materials and Methods. The unpulsed target group was labeled with a low CFSE concentration and was incubated without peptide. The VSV peptide-pulsed target group was

labeled with a high CFSE concentration and was incubated with VSV peptide. The two groups were pooled and *i.v.* injected into recipient mice that had been infected with VSV 7 days earlier. 16 hours after transfer of donor cells, splenocytes were purified from the infected recipient mice and cell killing of target cells was determined by flow cytometry (**Fig. 4.6A**). The calculated % Specific Lysis was decreased ICAM-$1^{-/-}$$CD28^{-/-}$ mice, suggesting that deficiency in both ICAM-1 and CD28 impaired CTL cytotoxic ability (**Fig. 4.6B**).

To evaluate the memory CD8+ T cell responses, mice were again infected with VSV and the % of memory CD8+ T cells specific for VSV peptide was determined 70 days post-infection. This should be a sufficient amount of time for the acute response to occur, for effector CTLs to die, and for differentiation of memory CD8+ T cells. **Figure 4.7** shows the results for C57Bl/6, ICAM-$1^{-/-}$, and ICAM-$1^{-/-}$$CD28^{-/-}$ mice. As expected, the percentage of memory CD8+ T cells specific for VSV peptide is lower than the percentage of effector CD8+ T cells that had been specific for VSV peptide (**Fig. 4.4**), since fewer numbers of memory cells are needed for surveillance than the number of effector cells needed to combat an acute infection. Similar to the results of the effector CD8+ T cells (**Fig. 4.4**), ICAM-$1^{-/-}$ mice had a stronger response than C57Bl/6 mice and ICAM-$1^{-/-}$$CD28^{-/-}$ mice. In contrast, ICAM-$1^{-/-}$$CD28^{-/-}$ mice had a slightly elevated response compared to C57Bl/6 mice, although differences were not statistically significant. The memory response of adoptively transferred T cells from each group of mice was also examined (**Fig. 4.8**). In this case, ICAM-$1^{-/-}$$CD28^{-/-}$ transferred CD8+ T cells had the greatest IFN-γ response, although there was much variability among mice in this experiment and differences between groups were not statistically significant. The results from the memory experiments suggest that there may be less dependence on ICAM-1 and CD28 signaling in either the differentiation of memory CD8+ T cells or the initiation of memory responses.

Table 4.1. Antibodies used in this study and the concentrations at which they were tested to attempt to induce T_{reg} differentiation. All antibodies are anti-mouse antibodies with the exceptions of the anti-human antibody clones OKT3 and R6.5D6.

Table 4.1

Antibody Clone	Concentrations Tested (μg/mL)
Anti-CD3	
OKT3	0.5
145-2C11	0.01, 0.05, 0.1, 0.5, 1, 5
KT3	0.5
500A2	0.01, 0.05, 0.1, 0.5
C363.29B	0.5
Anti-ICAM-1	
R6.5D6	10
3E2	10, 20
166623	1, 20
YN1/1.7.4	10
BE2961	10, 20
3E2B	10, 20
KAT-1	10
Anti-CD28	
37.51	2.5

Figure 4.1. Stimulation of mouse total T cells through CD3+ICAM-1 does not appear to increase proliferation. Total T cells were isolated from C57Bl/6 mouse spleens, stained with CFSE, and stimulated *in vitro* using anti-CD3 (clone 500A2, used at 0.5 μg/mL) alone or in combination with anti-ICAM-1 (clones KAT-1 or YN1/1.7.4, used at 10 μg/mL) or anti-CD28 (clone 37.51, used at 2.5 μg/mL). Proliferation was assessed at Day 5 of stimulation by CSFE dilution. Representative of one experiment using mouse C57Bl/6 total T cells.

Figure 4.1

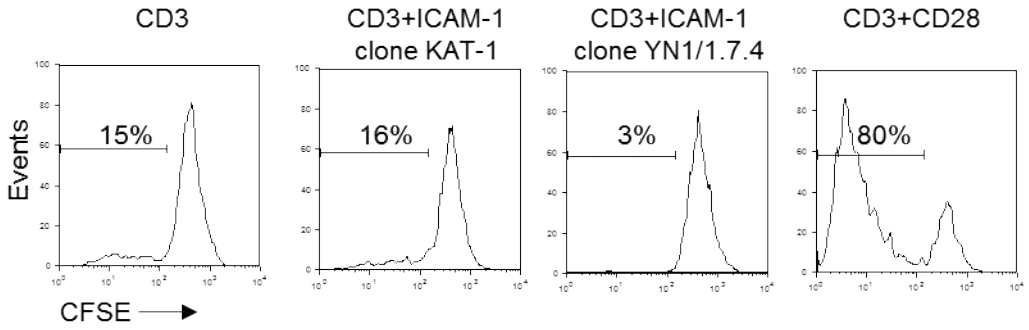

CD3
CD3+ICAM-1
clone KAT-1
CD3+ICAM-1
clone YN1/1.7.4
CD3+CD28
Events
15%
16%
3%
80%
CFSE

Figure 4.2. Mouse CD4+ T cells stimulated through CD3+ICAM-1 do not differentiate to a T_{reg} phenotype unless exogenous cytokines are added. (A) C57Bl/6 CD4+ T cells were stimulated as indicated in the absence of exogenous cytokines for 5 days and analyzed by flow cytometry. Representative of 7 experiments for anti-ICAM-1 clone KAT-1 and 3 experiments for clone YN1/1.7.4. Representative of a total of 18 experiments for all anti-ICAM-1 clones tested. (B) C57Bl/6 CD4+ T cells were stimulated as indicated for 5 days in the presence of the exogenous cytokines TGF-β1 and IL-2. Representative of 2 experiments for anti-ICAM-1 clone KAT-1 and 3 experiments for clone YN1/1.7.4.

Figure 4.2

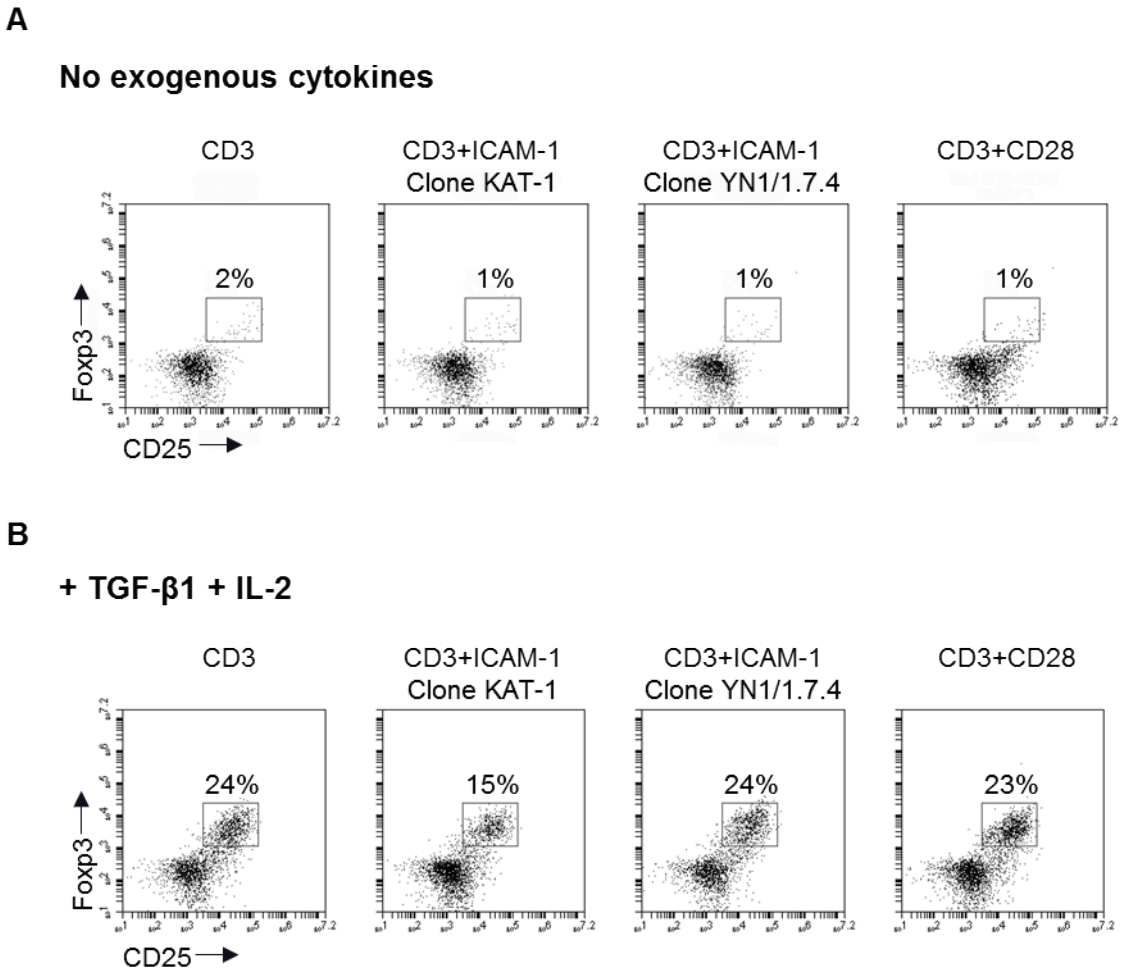

A
No exogenous cytokines
CD3
CD3+ICAM-1
Clone KAT-1
CD3+ICAM-1
Clone YN1/1.7.4
CD3+CD28
2%
1%
1%
1%
Foxp3
CD25
B
+ TGF-β1 + IL-2
CD3
CD3+ICAM-1
Clone KAT-1
CD3+ICAM-1
Clone YN1/1.7.4
CD3+CD28
24%
15%
24%
23%
Foxp3
CD25

Figure 4.3. Mouse CD4+ T cells stimulated through CD3+ICAM-1 are not suppressive. Suppression assays were performed using total T cells isolated from splenocytes as responder cells, and cells previously stimulated using the indicated stimulation treatments as potential suppressor cells. CD4+ T cells from mouse spleens were stimulated for 5 days using anti-CD3 (clone 500A2, used at 0.5 μg/mL) plus either anti-ICAM-1 (clone YN1/1.7.4, used at 10 μg/mL) or anti-CD28 (used at 2.5 μg/mL). Where indicated, the cytokines TGF-β1 and IL-2 were added to cultures. After 5 days of stimulation the cells were spun over Lympholyte-M to remove dead cells, and the recovered live cells were added to the CFSE-labeled responder cells at a 1:2 Stimulated:Responder cell ratio. In some experiments, the actual ratio of Stimulated:Responder cells used was less than 1:2. The co-cultured cells were stimulated using anti-CD3 plus anti-CD28 antibodies for 3-5 days and proliferation of the responder cells was analyzed using flow cytometry. Representative of 4 experiments.

Figure 4.3

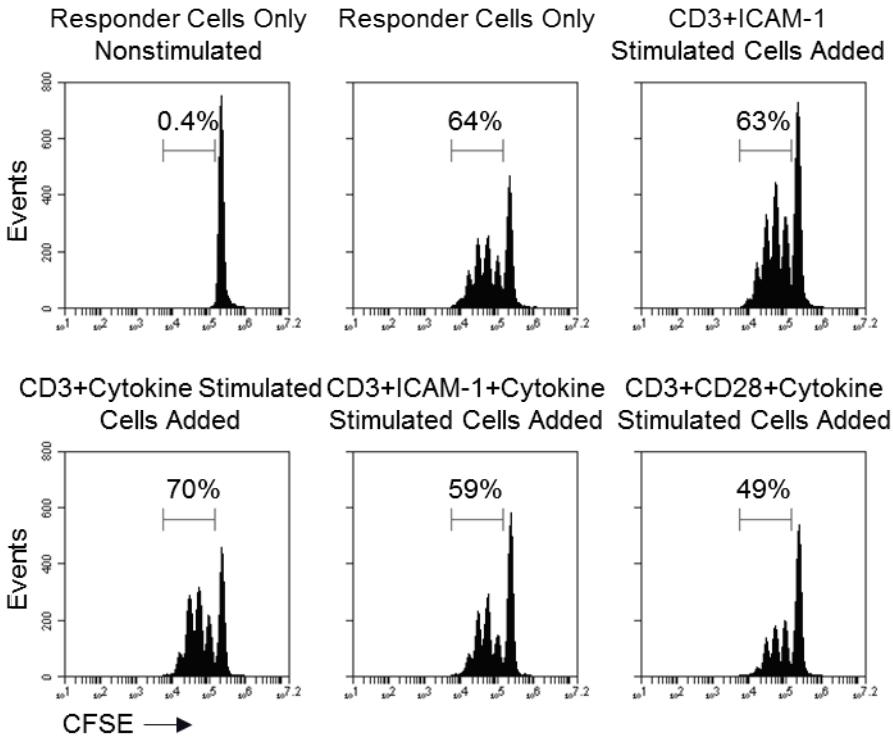

Responder Cells Only
Nonstimulated
Responder Cells Only
CD3+ICAM-1
Stimulated Cells Added
0.4%
64%
63%
Events
CD3+Cytokine Stimulated
Cells Added
CD3+ICAM-1+Cytokine
Stimulated Cells Added
CD3+CD28+Cytokine
Stimulated Cells Added
70%
59%
49%
Events
CFSE

Figure 4.4. CD8+ T cells from ICAM-1-deficient and ICAM-1/CD28-double deficient mice produce IFN-γ after VSV infection. (A) C57Bl/6, ICAM-$1^{-/-}$, and ICAM-$1^{-/-}$$CD28^{-/-}$ mice were infected *i.v.* with $1x10^6$ PFU of VSV. Seven days after infection, splenocytes were isolated from infected mice and cultured for 5-6 hours in the presence or absence of VSV peptide specific for MHC Class I as described in the Materials and Methods and Appendix Protocol 8. After incubation, the splenocytes were stained for CD8, F4/80, and intracellularly for expression of IFN-γ and analyzed by flow cytometry. Macrophages were excluded from the analysis by gating out the F4/80+ cells. The percentage shown in the upper right quadrants represents the percentage of CD8+ T cells that were IFN-γ+. This value was calculated as: [# IFN-γ+CD8+ cells/(# IFN-γ+CD8+ cells + # IFN-γ(-)CD8+ cells)] x 100%. Representative of 3-8 mice per group. (B) The data in panel (A) are shown as the mean +/- SEM for each group. One-way ANOVA and Tukey's Multiple Comparison post-test were performed to compare the results from each group of mice. The asterisk indicates a statistically significant difference between the percentage of CD8+ cells producing IFN-γ in the splenocyte culture from ICAM-$1^{-/-}$ mice compared to ICAM-$1^{-/-}$$CD28^{-/-}$ mice (* $p < 0.05$).

Figure 4.4.

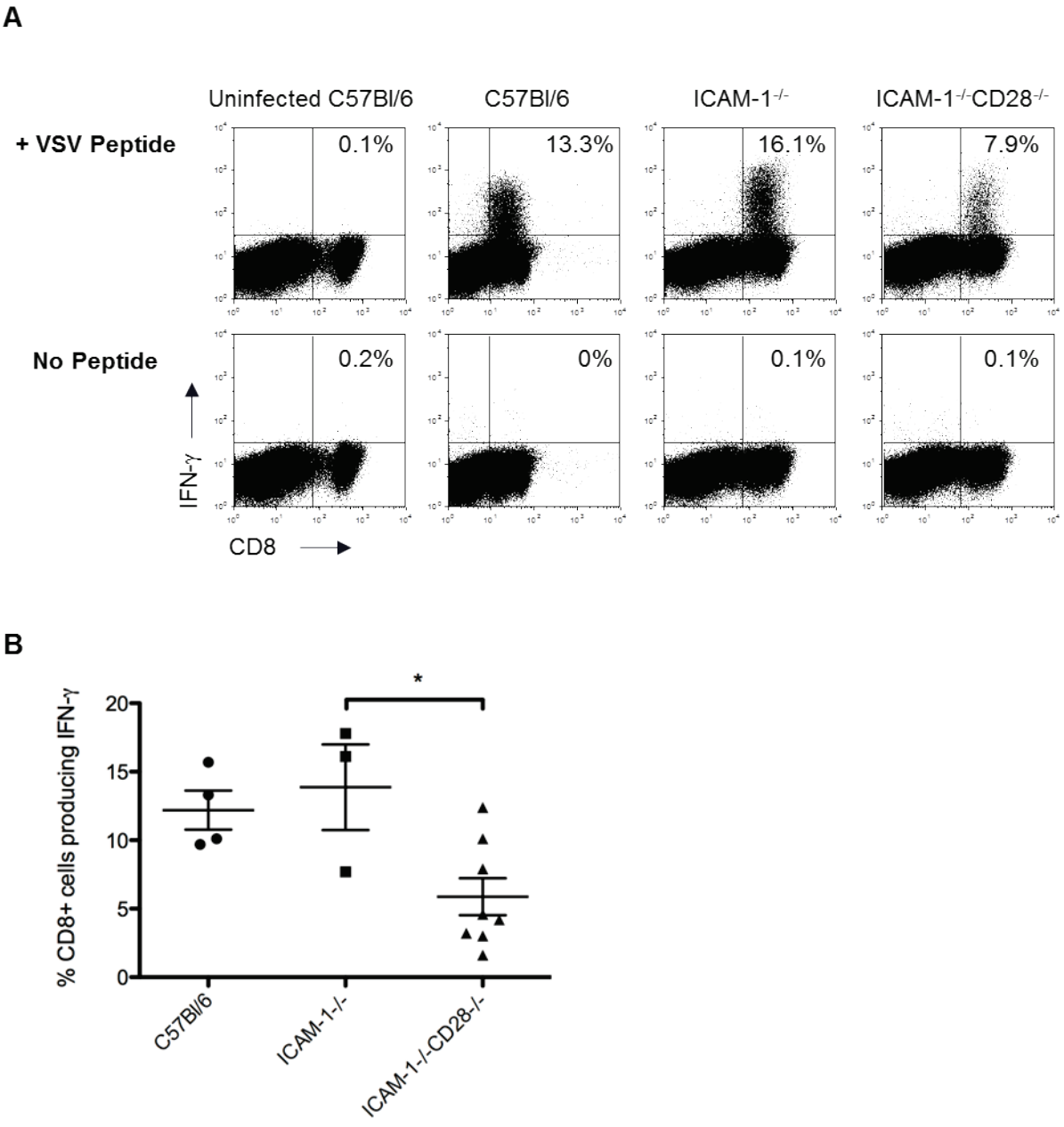

A
Uninfected C57Bl/6
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-
+ VSV Peptide
No Peptide
0.1%
13.3%
16.1%
7.9%
0.2%
0%
0.1%
0.1%
IFN-γ
CD8
B
% CD8+ cells producing IFN-γ
20
15
10
5
0
*
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-

Figure 4.5. Adoptively transferred T cells from ICAM-1-deficient and ICAM-1/CD28-double deficient mice produce IFN-γ after VSV infection. (A) T cells were purified from C57Bl/6, ICAM-$1^{-/-}$, and ICAM-$1^{-/-}$CD$28^{-/-}$ mice and transferred by *i.v.* injection into TCR$\beta^{-/-}$ mice. Four weeks after adoptive T cell transfer, these TCR$\beta^{-/-}$ recipient mice were infected *i.v.* with $1x10^6$ PFU of VSV. Seven days after infection, splenocytes were isolated from infected mice and cultured for 5-6 hours in the presence or absence of VSV peptide specific for MHC Class I. After incubation, the splenocytes were stained for CD8, F4/80, and intracellularly for expression of IFN-γ and analyzed by flow cytometry. Macrophages were excluded from the analysis by gating out the F4/80+ cells. The percentage shown in the upper right quadrants represents the percentage of CD8+ T cells that were IFN-γ+. Representative of 4-7 mice per group. (B) The data in panel (A) are shown as the mean +/- SEM for each group. One-way ANOVA and Tukey's Multiple Comparison post-test were performed to compare the results from each group of mice. There were no statistically significant differences among the groups.

Figure 4.5

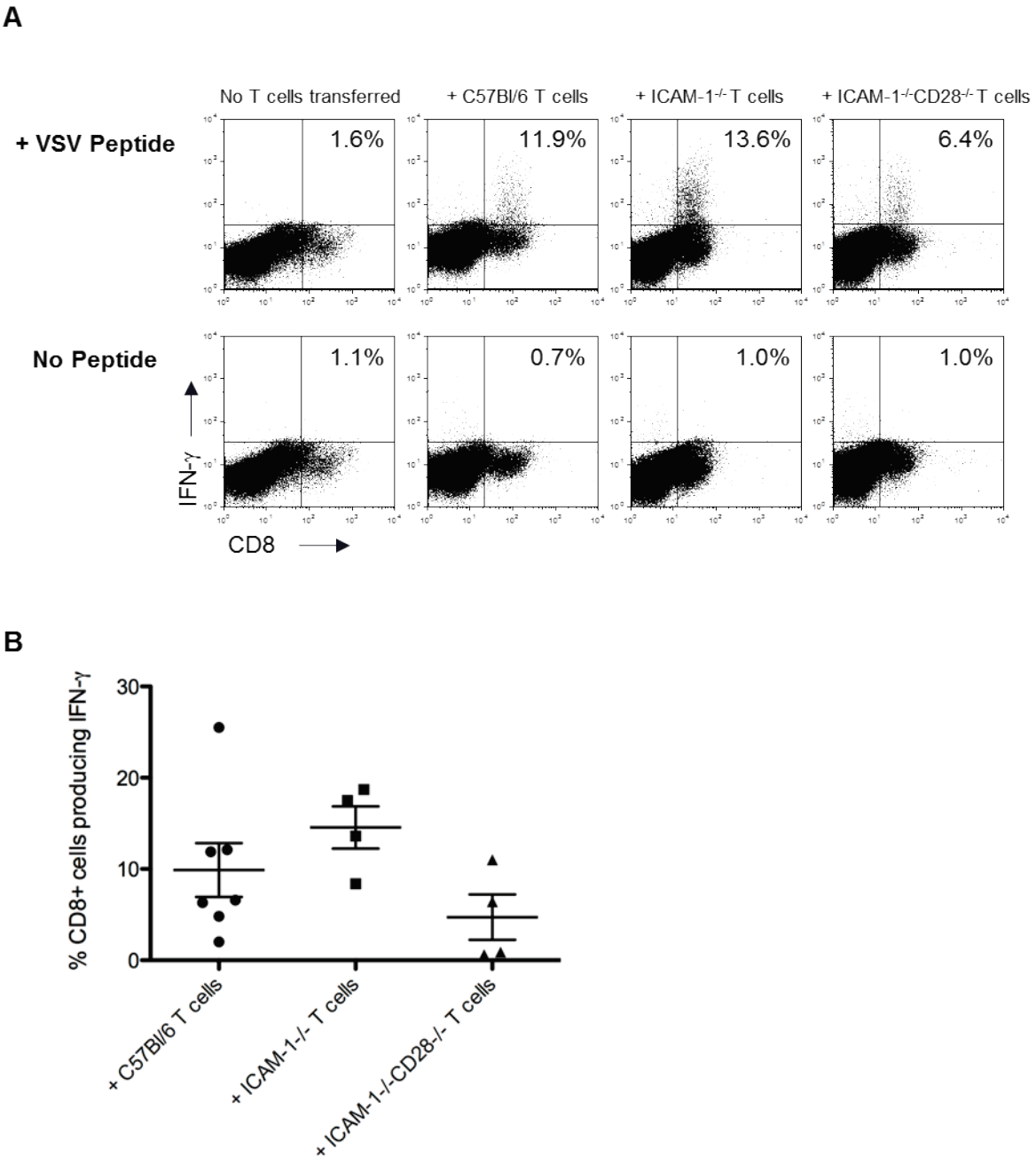

A
No T cells transferred
+ C57Bl/6 T cells
+ ICAM-1-/- T cells
+ ICAM-1-/-CD28-/- T cells
+ VSV Peptide
No Peptide
1.6%
11.9%
13.6%
6.4%
1.1%
0.7%
1.0%
1.0%
IFN-γ
CD8
B
% CD8+ cells producing IFN-γ
30
20
10
0
+ C57Bl/6 T cells
+ ICAM-1-/- T cells
+ ICAM-1-/-CD28-/- T cells

Figure 4.6. CD8+ T cells from ICAM-1-deficient and ICAM-1/CD28-double deficient mice have cytolytic function *in vivo*. (A) *In vivo* cytotoxicity assays were performed as described in the Materials and Methods and Appendix Protocol 12. $CFSE^{lo}$ cells represent control splenocytes and $CFSE^{hi}$ cells represent splenocytes loaded with VSV peptide. $CFSE^{lo}$ and $CFSE^{hi}$ cells from uninfected donor mice were *i.v.* injected into VSV-infected mice at a 1:1 ratio. Sixteen hours after cell transfer, splenocytes were isolated from the mice and the proportion of donor cells killed by the recipient mouse's immune system was determined by flow cytometry. Before analysis, autofluorescent cells were gated out, and PKH26+ transferred cells were gated on to give the plots shown in (A). Percentages of cells in the $CFSE^{lo}$ and $CFSE^{hi}$ peaks are shown. Representative of 3-4 mice per group. (B) The data in panel (A) were used to calculate the % Specific Lysis as described in the Materials and Methods. Results are shown as the mean +/- SEM for each group. One-way ANOVA and Tukey's Multiple Comparison post-test were performed to compare the results from each group of mice. The asterisks indicate statistically significant differences between the percent specific lysis by C57Bl/6 mice compared to ICAM-$1^{-/-}CD28^{-/-}$ mice (* $p < 0.05$), and the percent specific lysis by ICAM-$1^{-/-}$ mice compared to ICAM-$1^{-/-}CD28^{-/-}$ mice (* $p < 0.05$).

Figure 4.6

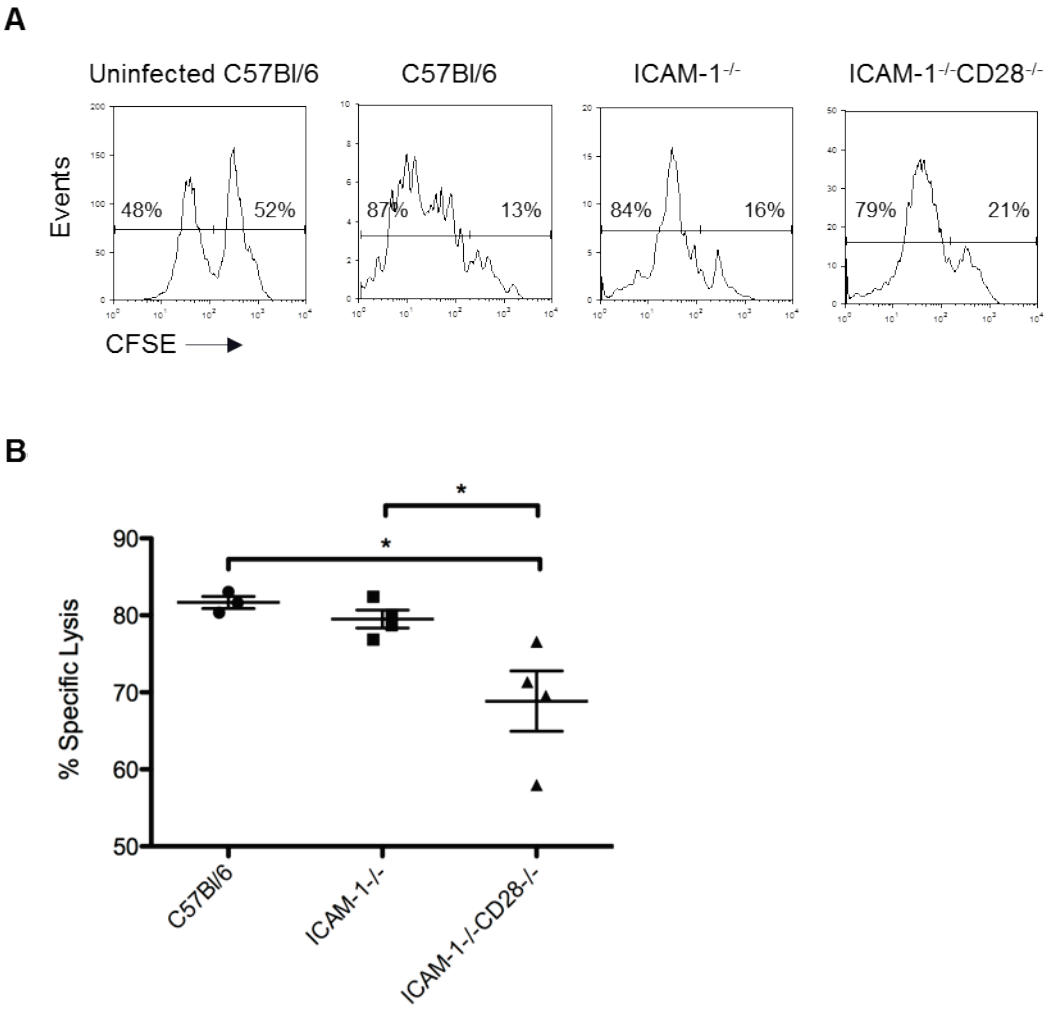

A
Uninfected C57Bl/6
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-
Events
48%
52%
87%
13%
84%
16%
79%
21%
CFSE
B
% Specific Lysis
90
80
70
60
50
*
*
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-

Figure 4.7. A CD8+ T cell memory response is generated in ICAM-1-deficient and ICAM-1/CD28-double deficient mice after VSV infection. (A) C57Bl/6, ICAM-$1^{-/-}$, and ICAM-$1^{-/-}$ $CD28^{-/-}$ mice were infected *i.v.* with $1x10^6$ PFU of VSV. **Seventy** days after infection, splenocytes were isolated from infected mice and cultured and cultured for 5-6 hours in the presence or absence of VSV peptide specific for MHC Class I. After incubation, the splenocytes were stained for CD8, F4/80, and intracellularly for expression of IFN-γ and analyzed by flow cytometry. Macrophages were excluded from the analysis by gating out the F4/80+ cells. The percentage shown in the upper right quadrants represents the percentage of CD8+ T cells that were IFN-γ+. Representative of 3-4 mice per group. (B) The data in panel (A) are shown as the mean +/- SEM for each group. One-way ANOVA and Tukey's Multiple Comparison post-test were performed to compare the results from each group of mice. The asterisks indicate statistically significant differences between the percentage of CD8+ cells producing IFN-γ in the splenocyte culture from C57Bl/6 mice compared to ICAM-$1^{-/-}$ mice (*** $p < 0.001$), and the percentage of CD8+ cells producing IFN-γ in the splenocyte culture from ICAM-$1^{-/-}$ mice compared to ICAM-$1^{-/-}$$CD28^{-/-}$ mice (** $p < 0.01$).

Figure 4.7

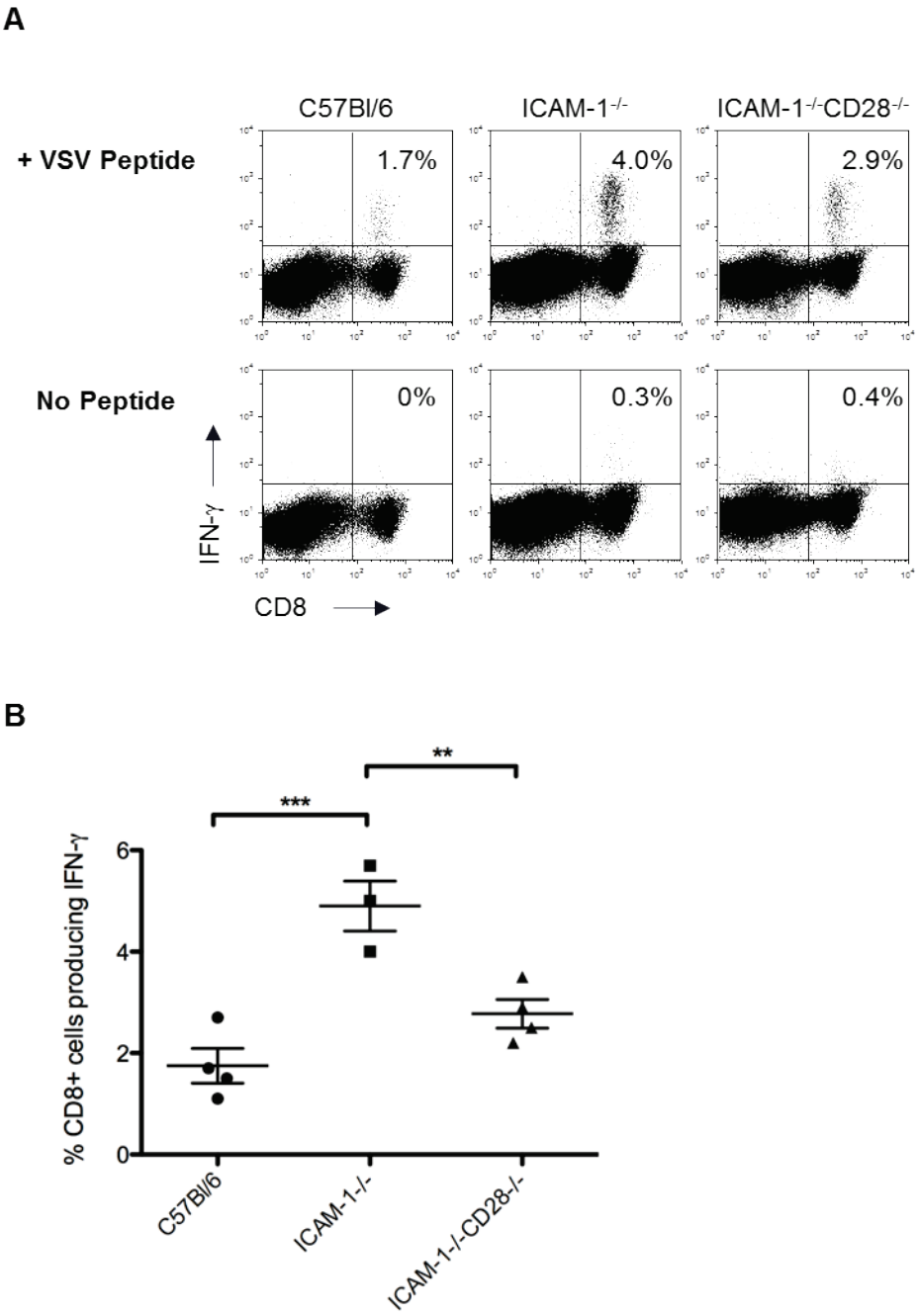
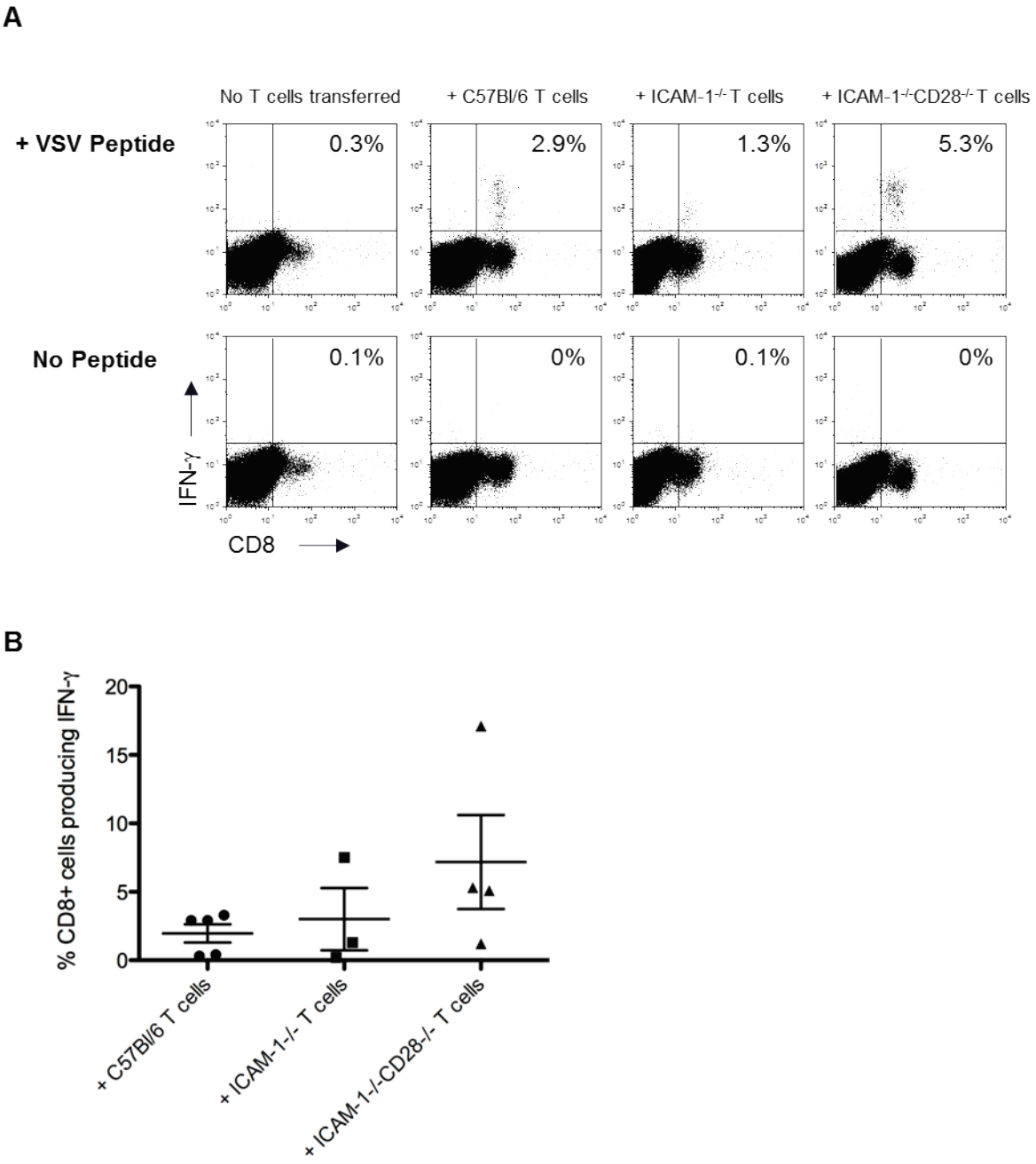

A
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-
+ VSV Peptide
No Peptide
1.7%
4.0%
2.9%
0%
0.3%
0.4%
IFN-γ
CD8
B
***
**
% CD8+ cells producing IFN-γ
0
2
4
6
C57Bl/6
ICAM-1-/-
ICAM-1-/-CD28-/-

Figure 4.8. A CD8+ T cell memory response is generated after VSV infection in TCR-$\beta^{-/-}$ mice with adoptively transferred T cells from ICAM-1 and ICAM-1/CD28 double deficient mice. (A) T cells were purified from C57Bl/6, ICAM-$1^{-/-}$, and ICAM-$1^{-/-}$CD$28^{-/-}$ mice and transferred by *i.v.* injection into TCR$\beta^{-/-}$ mice. Four weeks after adoptive T cell transfer, these TCR$\beta^{-/-}$ recipient mice were infected *i.v.* with $1x10^6$ PFU of VSV. **Seventy** days after infection, splenocytes were isolated from infected mice and cultured for 5-6 hours in the presence or absence of VSV peptide specific for MHC Class I. After incubation, the splenocytes were stained for CD8, F4/80, and intracellularly for expression of IFN-γ and analyzed by flow cytometry. Macrophages were excluded from the analysis by gating out the F4/80+ cells. The percentage shown in the upper right quadrants represents the percentage of CD8+ T cells that were IFN-γ+. Representative of 3-5 mice per group. (B) The data in panel (A) are shown as the mean +/- SEM for each group. One-way ANOVA and Tukey's Multiple Comparison post-test were performed to compare the results from each group of mice. There were no statistically significant differences among the groups.

Figure 4.8


A
No T cells transferred
+ C57Bl/6 T cells
+ ICAM-1-/- T cells
+ ICAM-1-/-CD28-/- T cells
+ VSV Peptide
No Peptide
0.3%
2.9%
1.3%
5.3%
0.1%
0%
0.1%
0%
IFN-γ
CD8
B
% CD8+ cells producing IFN-γ
20
15
10
5
0
+ C57Bl/6 T cells
+ ICAM-1-/- T cells
+ ICAM-1-/-CD28-/- T cells

DISCUSSION

Following our studies using human naïve CD4+ T cells, we asked if costimulation through ICAM-1 yielded similar differentiation when using mouse CD4+ T cells. Activation and differentiation might be predicted to be similar since the extracellular domains of human and mouse ICAM-1 are similar. However, there could be differences due to interspecies variations such as the nonconserved regions in the cytoplasmic domain of ICAM-1. We found that while costimulation through ICAM-1 in human naïve CD4+ T cells induces differentiation to T_{reg} and memory cells, costimulation through ICAM-1 on mouse CD4+ T cells did not yield inducible T_{reg} cells (**Fig. 4.2A**). These cells neither expressed Foxp3 nor lead to suppression of responder T cell proliferation (**Fig. 4.2A, Fig. 4.3**).

The differences we observed in T_{reg} induction can be speculated to be due to several factors. First, the differences in the intracellular domain of ICAM-1 between human and mouse species may determine whether downstream signaling molecules can cause differentiation. Secondly, the *in vitro* system of stimulating ICAM-1 using plate-bound antibodies against ICAM-1 has limitations. Even though we tried six clones of commercially available anti-mouse ICAM-1 and one clone of anti-human ICAM-1, we did not observe increased proliferation or differentiation to T_{reg} cells (**Fig. 4.1, 4.2A**). It is possible that the antibodies we used did not bind to motifs on ICAM-1 that would generate the proper signal or signal strength needed for activation or differentiation. Lastly, there could be other yet unidentified differences between human and mouse ICAM-1 function that led to the differences we observed.

Although many characteristics of the immune system are the same in humans and mice, there are differences that have been identified as well (reviewed in (34)). For example, when comparing expression of CD28 on mouse and human T cells, almost all mouse T cells express

CD28, while only 80% of human CD4+ T cells and 50% of human CD8+ T cells express CD28. After activation, human T cells but not mouse T cells, can express MHC Class II. In the EAE mouse model for multiple sclerosis, blocking IFN-γ protects mice from the disease, but blocking IFN-γ actually worsens the disease in patients with MS. Unfortunately, mouse studies of the immune system do not always directly translate to human studies.

Despite our lack of evidence that mouse ICAM-1 is involved in T_{reg} induction, there are some hints from the literature that ICAM-1 may be involved in T_{reg} function. ICAM-1 is expressed at higher levels on T_{reg} cells versus non-T_{reg} cells in both mice and humans (35, 36). *Icam1*^{tm1Jcgr} mice have fewer T_{reg} cells in peripheral organs and a heightened immune response to infection with *Mycobacterium tuberculosis* (7). Mice deficient in an ICAM-1 ligand, LFA-1, have an approximately 50% decrease in T_{reg} numbers, decreased T_{reg} suppressor function, and show an impairment in inducible T_{reg} differentiation (37).

The effect of removing a particular costimulatory molecule is often dependent upon the mouse strain or disease studied. For example, CD28-deficient mice have a reduced immune response to some autoimmune diseases, but a heightened immune response to diabetes in the NOD model (38). As another example, C57Bl/6 mice deficient in ICAM-1 due to the *Icam1*^{tm1Jcgr} mutation have an increased immune response to *M. tuberculosis* infection (7), but are protected from developing diabetes when crossed with an NOD mouse strain (39). In addition, the outcome of EAE induction differed significantly between the *Icam1*^{tm1Jcgr} strain and the *Icam1*^{tm1Bay} strain (8).

As mentioned above, it has been previously reported that ICAM-1 mutant mice have similar percentages of T_{reg} cells in the thymus, but lower percentages of T_{reg} cells in the periphery than wild-type mice (7, 40). The project studying the effects of ICAM-1 and CD28

deficiency on the efficacy of anti-viral responses was performed before our project to study T_{reg} differentiation began, and before reagents to identify T_{reg} cells were commercially available. Therefore, we do not know whether the sometimes heightened CD8+ T cell response to VSV seen in the *Icam1*^{tm1Jcgr} strain was influenced by a deficiency in T_{reg} numbers or function.

Our results looking at IFN-γ production by activated CD8+ T cells demonstrates the progression of an anti-viral immune response. Endogenously there are a low percentage of CD8+ T cells specific for a viral antigen, such as the VSV peptide used in our study. After infection, these virus-specific CD8+ T cells become activated and undergo clonal expansion to generate a population of cells capable of producing IFN-γ (**Fig. 4.4, 4.5**) and causing cytolysis of target cells (**Fig. 4.6**). After the infection has been cleared, memory CD8+ T cells remain that are capable of responding to a second challenge with the same infective agent (**Fig. 4.7, 4.8**). Of course, other cells and cytokines are involved in the anti-viral response, but we chose to focus on CD8+ T cells for our initial studies.

ICAM-1 has multiple roles in the immune system as an adhesion molecule, a costimulatory ligand on APCs, and as a costimulatory molecule on human T cells. Our data in Part I suggest that differences may exist between the ability of ICAM-1 on human T cells and mouse T cells to influence differentiation. Results in Part II show that ICAM-1 and CD28 play a role in the immune response to VSV, but are not required for the CD8+ T cell response. A better understanding of costimulatory molecule function during immune responses will aid in designing effective therapies, such as improved vaccines or treatments for viral infections.

CHAPTER 4 ACKNOWLEDGEMENTS

In Part I of this Chapter, I would like to acknowledge the collaboration of former graduate students Dr. Abby Dotson and Courtney Gdowski. Abby collaborated with me in testing the different antibody clones by studying expression of the activation marker CD69 and differentiation of mouse CD4+ T cells to central memory T cells. Courtney assisted with the mouse T cell suppression assays and some of the experiments to detect differentiation or expansion of T_{reg} cells. In Part II of this Chapter, I would like to acknowledge the substantial work of former graduate student Dr. Jake Kohlmeier. Jake did the initial work to begin this project, such as crossing and genotyping the ICAM-$1^{-/-}$$CD28^{-/-}$ double knockout mice and determining the VSV titer. He performed the initial experiments using VSV infected mice and trained me in the procedures so I could finish the remaining experiments after his departure from KU. I would also like to thank the Animal Care Unit, especially Jodi Troup, for training and assistance. The project to study differentiation of mouse CD4+ T cells to T_{reg} cells was supported by grants from the Great Plains Diabetes Institute and the KU BIO Center.

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CHAPTER 5

THE SPECIFIC COSTIMULATORY SIGNAL CAN INFLUENCE NAÏVE CD4+ T CELL ACTIVATION AND DIFFERENTIATION OUTCOME

ABSTRACT

The two-signal hypothesis asserts that an antigenic signal plus a costimulatory signal must be received by a naïve T cell for full activation and differentiation to occur. Naïve T cells undergo anergy or apoptosis after receipt of a signal through the TCR alone (signal 1) without also receiving a costimulatory signal (signal 2). While the best studied costimulatory molecule is CD28, many other proteins on the T cell surface are capable of delivering costimulatory signals. Our lab has previously shown that Intercellular Adhesion Molecule-1 (ICAM-1), when expressed on the naïve CD4+ T cell surface, can function as a costimulatory molecule to promote T cell activation and differentiation. In this chapter, we investigated some differences in signaling and functional outcome after costimulation through ICAM-1 compared to costimulation through CD28.

INTRODUCTION

Naïve T cell differentiation is thought to be influenced by several factors including 1) the strength of the TCR signal, 2) polarizing cytokines, and 3) costimulatory molecules expressed on the T cell and APC (1-3). Costimulatory signals 1) enhance cellular proliferation, 2) increase IL-2 production, 3) promote cell survival, and 4) induce cellular differentiation (4). TCR signaling plus costimulation activates several intracellular signaling pathways to induce gene expression, including the NF-κB, NFAT, AP1, and MYC pathways (5). These changes in gene expression influence both activation and regulation of the immune response (6). In the absence of costimulation, the T cell becomes anergic or apoptotic as a mechanism of immune tolerance.

While the classic costimulatory molecule is CD28, alternate costimulatory molecules include CD2, CD5, CD9, CD27, CD44, CD46, CD81, LFA-1, VLA-4, OX40, 4-1BB, CD40L, LIGHT, SLAM, ICOS, and the negative regulatory proteins CTLA-4 and PD-1 (4, 7). In addition, our lab previously determined that ICAM-1 on the T cell surface could function as a costimulatory molecule (8). Some costimulatory molecules such as CD28, LFA-1 (9), and ICAM-1 (10) are expressed on naïve T cells. In contrast, most members of the Tumor Necrosis Factor Receptor (TNFR) family, such as OX40, 4-1BB, and CD40L, are not expressed on naïve T cells, but rather are upregulated upon T cell activation.

There are differing theories regarding the precise function of costimulatory molecules. Some studies suggest that costimulatory molecules simply enhance TCR signaling and do not deliver unique signals. One study showed that increasing the dose of antigenic stimulus plus costimulation led to changes in the cytokines that T cells secreted, suggesting that costimulatory molecules can lower the threshold for activation, but do not favor distinct differentiation pathways themselves (11). Another study found that CD28 signals through some of the same

molecules as the TCR, and that costimulation through CD28 will increase or decrease expression of genes induced by TCR stimulation, but not induce new genes (5).

However, other results argue in favor of differences between costimulatory molecules and a role greater than simply enhancing TCR signaling. Our lab and others have showed that signaling through CD28 can favor T_{H2} cytokines and maintain T_{H1} cytokine signaling, while signaling through LFA-1 can favor T_{H1} cytokines (3, 12). Our lab also found differences in cytokine secretion and cell survival among T cells costimulated by CD28, LFA-1, or ICAM-1 (8, 10). Signaling differences have been demonstrated between the Tumor Necrosis Factor Receptor (TNFR) family members that are known to signal through TNF receptor-associated factor (TRAF) adaptor proteins, and CD28 which is not associated with TRAF (13). Signaling differences were also shown between human naïve CD4+ T cells costimulated through CD28 or CD2, since it was shown that CD28 signaling led to greater NF-κB phosphorylation, while CD2 signaling led to greater S6-ribosomal protein phosphorylation (14). From the perspective of the APC, dendritic cells (DC) can be classified by how they favor different types of T cell responses. DC1 cells polarize induce differentiation of T_{H1} cells, DC2 cells induce T_{H2} cells, and regulatory DC cells induce T_{reg} cells due to the cytokines produced and coreceptors expressed by each dendritic cell subset (2, 15).

In this chapter, we compare signaling and functional outcomes between the costimuli ICAM-1 and CD28. Although both are members of the Immunoglobulin Superfamily, there are many differences between these molecules that, in theory, could potentially lead to differences in signaling. CD28 is a transmembrane protein containing one extracellular immunoglobulin (Ig) domain, and is often expressed as a homodimer (5). The ligands of CD28, B7.1 (CD80) and B7.2 (CD86), are found on professional APCs. The intracellular tail of CD28 has been shown to

interact with PI3K, Vav1, ITK, TEC, Grb-2, and Lck (5). ICAM-1 is a transmembrane protein with 5 extracellular Ig domains, and is often expressed as a homodimer. The primary ligands of ICAM-1 are expressed on leukocytes and are LFA-1 (CD11a/CD18), Mac-1 (CD18/CD11b) and p150,95 (CD18/CD11c). ICAM-1 was found to associate laterally with IL-2Rα, and HLA Class I and II molecules in the HUT-102B2 T cell line and JY B cell line (16, 17). The proteins that interact with the ICAM-1 cytoplasmic tail are still being identified, although data from our lab suggests the association of signaling proteins such as Lck and Erk1/2 (18). Stimulating ICAM-1 on APCs has been shown to activate the RhoA family of G-proteins, Abl tyrosine kinase, and Src-family kinases (19).

Comparing costimulation through ICAM-1 and CD28 *in vitro* we show 1) the timing of activation and proliferation differ, 2) the cytokines and chemokines secreted differ, and 3) the kinases activated differ. The results presented in this chapter further support the hypothesis that the nature of the costimulatory molecule is a factor that influences the outcome of naïve CD4+ T cell stimulation.

MATERIALS AND METHODS

Cell Purification

Naïve CD4+ T cells were isolated from the peripheral blood of healthy donors using the same procedure described in the Materials and Methods in Chapter 2 and Appendix Protocol 1. Naïve CD4+ T cells for this study were defined as CD45RA+CD11a^{lo}CD27+ or CD45RO(-)CD11a^{lo}CD27+.

Cell Culture Reagents

Cell culture reagents used in this chapter are the same as those described in the Chapter 2 Materials and Methods.

Stimulating Antibodies

The antibody clones, antibody concentrations, method of adhering antibodies to the tissue-culture plates, and method of antibody-mediated stimulation of cells used in this chapter are the same as those described in the Chapter 2 Materials and Methods and Appendix Protocol 3.

Flow Cytometry Antibodies

Anti-CD25-TriColor was purchased from Caltag/Life Technologies (Carlsbad, CA).

Flow Cytometry Surface Staining

The flow cytometry staining procedure to detect cell-surface proteins is described in the Chapter 2 Materials and Methods and Appendix Protocol 4.

Flow Cytometry Analysis

Flow cytometry and data analysis were performed as described in the Chapter 2 Materials and Methods.

Luminex

Cell culture supernates were collected from stimulated cultures and used after clarification by centrifugation. Cytokine and chemokine production was measured using a Human Cytokine 25-Plex kit (Biosource/Life Technologies, Carlsbad, CA). The protocol followed was that supplied in the manufacturer's technical manual and used by Dr. Marcia Chan's lab at Children's Mercy Hospital in Kansas City, MO. A summary of the protocol will be provided in this chapter. First, the provided 96-well filter plate was washed with Wash Solution. All washes were performed using a vacuum manifold for aspiration. Next, the 25-plex antibody-coated beads were added to the plate, and the plate was washed twice with Wash Solution. Incubation Buffer was added to each well, the standards were added to the appropriate wells, Assay Diluent and the Samples were added to the appropriate wells, and the plate was incubated for 2 hours. The plate was then washed twice with Wash Solution. The biotinylated-detector antibodies were added and incubated for 1 hour, and the plate was washed twice with Wash Solution. Streptavidin-RPE was added and incubated 30 minutes, and the plate was washed three times using Wash Solution. Finally, 100 µL Wash Solution was added to each well. Cytokine concentrations were analyzed using a Luminex 200 (Luminex, Austin, TX) and STarStation 2.3 software (Applied Cytometry, Sheffield, UK).

Kinase Array

Cell lysates were prepared using Cell Lysis Buffer from Cell Signaling Technology (Danvers, MA) and the manufacturer's recommended lysis protocol. The cells analyzed were naïve CD4+ T cells that had been stimulated for 4.5 days in culture using either anti-CD3 plus anti-ICAM-1 antibodies or anti-CD3 plus anti-CD28 antibodies as previously described in the Chapter 2 Materials and Methods. To summarize the lysis procedure, stimulated cells were removed from the cell culture plate, pooled, centrifuged, and the supernate was aspirated. The cell pellets were resuspended in cold PBS plus Inhibitors solution (containing 0.1 M sodium orthovanadate and 0.5M EDTA), then centrifuged, and the supernate was aspirated. The cell pellet was resuspended in 100 µL Cell Lysis Buffer (Cell Signaling Technology) with 1 mM PMSF added, and incubated for 5 minutes on ice. Cell lysates were briefly vortexed, then centrifuged for 10 minutes at 4°C, and supernates were collected. Next, 100 µL Lysis Buffer was added to the pellet, vortexed briefly, and incubated for 5 minutes on ice. This second step cell lysate was centrifuged for 10 minutes at 4°C, and the supernate was added to the supernate collected after the first lysis. Cell lysates were stored at -70°C. Before use, the lysate protein concentrations were determined using a standard Bradford Assay. Activated kinases were detected using Tyrosine Kinase Substrate I CelluSpots Kinase Substrate Arrays (Intavis Bioanalytical Instruments AG, Koeln, Germany) and a Phos-Tag 300/460 Phosphoprotein Blot Stain kit (Perkin Elmer Life and Analytical Sciences, Inc., Shelton, CT). The protocol used was that supplied in the manufacturers' technical manuals and used by Dr. Marcia Chan's Lab at Children's Mercy Hospital. To summarize the procedure, first, the arrays were blocked in Phos-Tag Blocking Buffer for 2 hours, then washed briefly with deionized water. Cell lysates were added to 10 µM ATP, 2.5 mM DTT, and HTScan Tyrosine Kinase Buffer (Cell Signaling

Technology) and this solution was added to the arrays and incubated for 1.5 hours. The arrays were washed twice in Phos-Tag Wash Buffer, followed by addition of the Phos-Tag Stain and incubation for 1 hour. The arrays were washed three times in Wash/Destain Buffer, then rinsed briefly in deionized water and allowed to air dry. The arrays were analyzed using a Storm 860 Molecular Imager (Molecular Dynamics/GE Healthcare Life Sciences, Pittsburgh, PA) with ImageQuant software (GE Healthcare Life Sciences).

Human Subjects

Peripheral blood was obtained after informed consent of healthy volunteers. Procedures were approved by The University of Kansas Institutional Review Board.

RESULTS

The kinetics of activation of naïve CD4+ T cells differ when stimulated through ICAM-1 compared with CD28

When performing experiments studying naïve CD4+ T cell activation and differentiation in our *in vitro* culture system, we observed that cells appeared to become activated faster and proliferate sooner when they were stimulated through CD3+CD28 compared to being stimulated through CD3+ICAM-1. Former graduate student Jake Kohlmeier had previously shown that total T cells costimulated through ICAM-1 had a delay in proliferation compared to total T cells costimulated through CD28 (20). To study the timing of activation of naïve CD4+ T cells after costimulation through ICAM-1 or CD28, we analyzed expression of the activation marker CD25 and proliferation over three time-points. As expected from previous data, we found that activation of naïve CD4+ T cells occurred sooner after costimulation through CD28 than through ICAM-1 (**Fig. 5.1A,B,C**). At Day 3 of stimulation, a greater proportion of cells expressed CD25 (**Fig. 5.1B**) and had divided at least once (**Fig. 5.1C**) when they were costimulated by CD28 compared with ICAM-1. The differences between cells stimulated through ICAM-1 and CD28 were not statistically significant at Days 5 and 7 of stimulation as the proportion of activated cells increased for both sets of stimuli.

The cytokines produced by naïve CD4+ T cells differ after costimulation through ICAM-1 compared with CD28

After naïve T cells undergo activation, they produce cytokines that help to control the immune response and chemokines that direct cell movement during immune responses. T cell subsets are often characterized by the types of cytokines they secrete. Some cytokines propagate an inflammatory response (e.g. IL-6, TNF-α), a T_{H1}/cellular immune response (*e.g.* IFN-gamma,

IL-12), or a T_{H2}/humoral immune response (*e.g.* IL-4, IL-5), while some suppress immune responses (*e.g*. IL-10, TGF-β1). Besides orchestrating cell migration, chemokines can also influence T cell differentiation either by acting on the APC or the T cell itself (21). We compared the cytokines and chemokines produced by naïve CD4+ T cells after costimulation through ICAM-1 with those produced after costimulation through CD28 using a multiplex Luminex assay. This particular Luminex kit allowed us to analyze 25 cytokines and chemokines simultaneously using the same cell culture supernate samples. It should be noted that this experiment was only performed once using cell culture supernates from one subject because the intention of this multiplex experiment was to provide preliminary data. This experiment was done in collaboration with Abby Dotson and with assistance from Dr. Marcia Chan and Nicole Gigliotti at Children's Mercy Hospital in Kansas City, MO.

The cytokines and chemokines we analyzed and their corresponding functions are listed in **Table 5.1** and are grouped by the classification scheme used by the company that manufactured the Luminex kit (*i.e.* Biosource). The graphs showing the concentration of each cytokine or chemokine versus the Day of Stimulation that the sample was collected are shown in **Figures 5.2-5.5**. Varying results were observed for each cytokine and chemokine studied. A summary of the results showing which cytokines had highest production after CD3+ICAM-1 stimulation, highest production after CD3+CD28 stimulation, similar production after each stimulation treatment, or were not produced or only produced in low amounts are listed in **Table 5.2**. Each stimulation treatment produced a variety of cytokines. CD3+ICAM-1 produced higher concentrations of some cytokines with immunosuppressive or anti-inflammatory properties (*e.g.* IL-10, IL-1R antagonist), while CD3+CD28 produced higher concentrations of

the pro-inflammatory cytokine IL-17. Both CD3+ICAM-1 and CD3+CD28 treatments produced T_{H1} (*e.g.* IFN-γ) and T_{H2} (*e.g.* IL-4 or IL-5) cytokines.

The kinases activated differ after costimulation through ICAM-1 compared with CD28

To compare what kinases are activated after costimulation through ICAM-1 or CD28, we used Kinase Substrate Arrays. These arrays consist of a 26 x 76 mm glass slide, containing 384 peptide substrates in duplicate (768 spots total) per slide. The peptide substrates are 15-mer peptides bound to cellulose by the C-terminus, with an acetylated N-terminus to increase stability. Each peptide substrate has a target kinase with specificity for that substrate. Therefore, solutions such as cell lysates containing kinases of interest can be screened. Detection occurs using a Phos-tag dye that binds to phosphoserine, phosphothreonine, and phosphotyrosine residues, and is attached to a fluorophore. This experiment was done in collaboration with Abby Dotson and with assistance from Dr. Marcia Chan and Nicole Gigliotti at Children's Mercy Hospital in Kansas City, MO. It should be noted that this experiment was only performed once, since it was intended to provide preliminary data.

When we compared lysates from cells stimulated through CD3+ICAM-1 with lysates from cells stimulated through CD3+CD28, we found some kinases that weren't activated (data not shown), some kinases that were activated in both cell samples, and some kinases that appeared to be specific to one of the stimuli (**Fig. 5.6, Table 5.3**). The stimulation time-point of 4.5 days was chosen because in previous differentiation experiments, this seemed to be a critical time-point where naïve CD4+ T cells that had been activated began to differentiate to effector, memory, and T_{reg} phenotypes. Some of the kinases detected were expected to be activated in stimulated T cells (*e.g.* Lck, Syk), while others were more unexpected (*e.g.* PDGFR, IGF1R).

The results from this kinase array experiment suggest some signaling pathways that might vary between ICAM-1 and CD28.

Figure 5.1. Naïve CD4+ T cells costimulated through CD28 undergo activation and proliferation sooner than naïve CD4+ T cells costimulated through ICAM-1. (A) Naïve CD4+ T cells were labeled with CFSE and stimulated for between 3-7 days. Cells were stained for expression of CD25 on the days of analysis. Representative of 3 experiments. (B) The kinetics of CD25 expression are shown as the means for 3 experiments ± SEM. The asterisk indicates a statistically significant difference between the percentage of CD25+ cells after costimulation through ICAM-1 and the percentage of CD25+ cells after costimulation through CD28 (paired t-Test, one tail $p<0.05$). (C) The kinetics of proliferation are shown as the means of 3 experiments ± SEM. The asterisks indicate a statistically significant difference between the percentage of cells that had divided after costimulation through ICAM-1 and the percentage of cells that had divided after costimulation through CD28 (paired t-Test, one tail $p<0.01$). There were no other significant differences.

Figure 5.1.

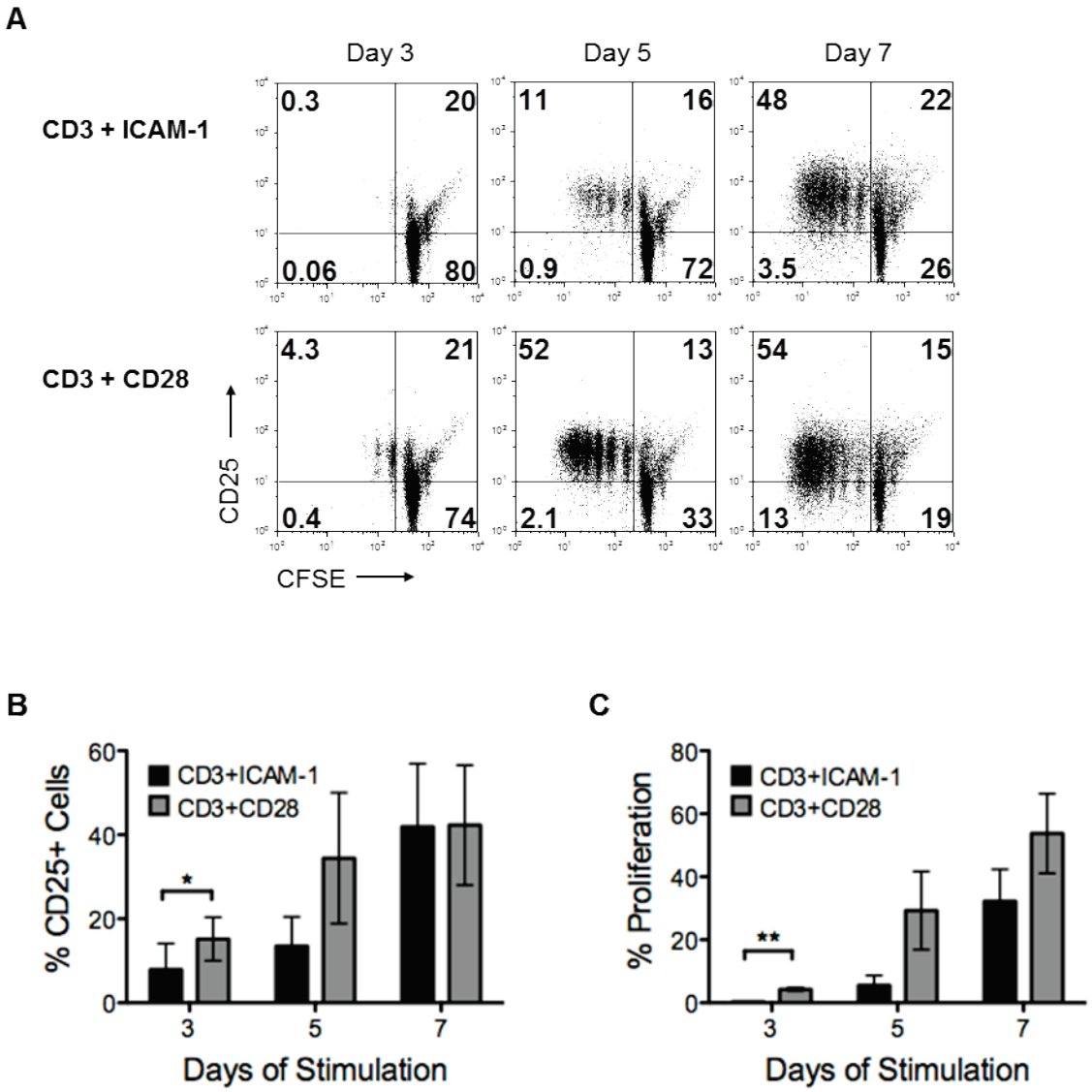
A
Day 3
Day 5
Day 7
CD3 + ICAM-1
CD3 + CD28
0.3
20
0.06
80
11
16
0.9
72
48
22
3.5
26
4.3
21
0.4
74
52
13
2.1
33
54
15
13
19
CD25
CFSE
B
% CD25+ Cells
CD3+ICAM-1
CD3+CD28
*
Days of Stimulation
C
% Proliferation
CD3+ICAM-1
CD3+CD28
**
Days of Stimulation

Table 5.1. The cytokines and chemokines analyzed in the Luminex assay are listed with their associated immunological functions (22, 23).

Table 5.1

Cytokine	Function
Inflammatory Panel	
GM-CSF	Growth of hematopoietic progenitor cells; differentiation of granulocytes and monocytes
IL-1β	Inflammation
IL-1 Receptor Antagonist	Anti-inflammatory
IL-6	Inflammation
IL-8 (CXCL8)	Migration of granulocytes and T cells; innate immunity; inflammation
TNF-α	Inflammation
Cytokine I Panel	
IFN-γ	Activation, growth, and differentiation of T cells, B cells, NK cells, and macrophages; T_{H1} response
IL-2	Growth and differentiation of T cells, B cells, and NK cells; T_{H1} response
sIL-2 Receptor	Detected in some cancers, infections, and autoimmune and inflammatory conditions; function unknown
IL-4	Growth of B cells, T cells, and monocytes; T_{H2} response
IL-5	Differentiation of eosinophils; T_{H2} response
IL-10	Immune suppression; T_{H2} response
Cytokine II Panel	
IFN-α	Resistance to viral infection
IL-7	Growth of T cell and B cell progenitors
IL-12	T_{H1} response
IL-13	T_{H2} response
IL-15	Growth and differentiation of T cells and NK cells
IL-17	Response to extracellular pathogens; autoimmunity; inflammation

Chemokine Panel	
Eotaxin (CCL11)	Migration of eosinophils, basophils, and mast cells; degranulation; T_{H2} response
IP-10 (CXCL10)	Migration of T cells; promotion of atherosclerosis; T_{H1} response
MCP-1 (CCL2)	Migration of monocytes, basophils, T cells, NK cells, and progenitor cells; CD4+ T cell differentiation; innate immunity; T_{H2} response
MIG (CXCL9)	Migration of T cells and progenitor cells; T_{H1} response
MIP-1α (CCL3)	Migration of monocytes, granulocytes, dendritic cells, T cells, B cells, NK cells, and progenitor cells; CD4+ T cell differentiation; innate immunity; T_{H1} response
MIP-1β (CCL4)	Migration of monocytes, dendritic, T cells, NK cells, and progenitor cells; innate immunity; T_{H1} response
RANTES (CCL5)	Migration of granulocytes, dendritic, T cells, and NK cells; innate immunity; T_{H1} and T_{H2} responses

Figure 5.2. Luminex "Inflammatory" Panel. Cell culture supernates were collected and analyzed by Luminex for the following analytes: A) GM-CSF, B) IL-1β, C) IL-1 Receptor Antagonist, D) IL-6, E) IL-8, F) TNF-α. Medium = complete RPMI medium, NS = nonstimulated (nonstimulated cell supernates were collected after 1 hour in culture). Data are the mean of duplicate samples from one experiment.

Figure 5.2

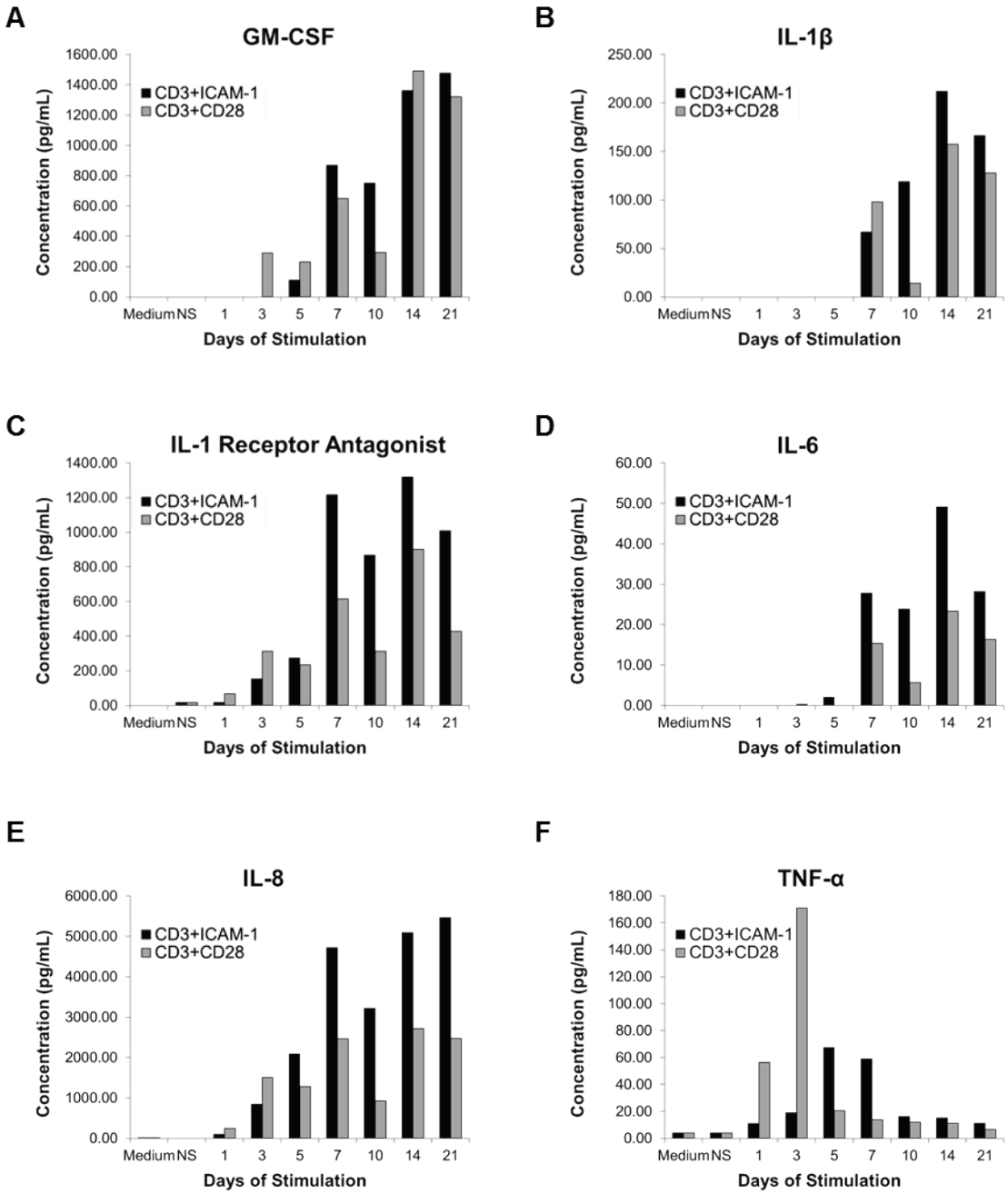
A
GM-CSF
Concentration (pg/mL)
1600.00
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
B
IL-1β
Concentration (pg/mL)
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
C
IL-1 Receptor Antagonist
Concentration (pg/mL)
1400.00
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
D
IL-6
Concentration (pg/mL)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
E
IL-8
Concentration (pg/mL)
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
F
TNF-α
Concentration (pg/mL)
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation

Figure 5.3. Luminex "Cytokine I" Panel. Cell culture supernates were collected and analyzed by Luminex for the following analytes: A) IFN-γ, B) IL-2, C) sIL-2R, D) IL-4, E) IL-5, F) IL-10. Medium = complete RPMI medium, NS = nonstimulated. Data are the mean of duplicate samples from one experiment.

Figure 5.3

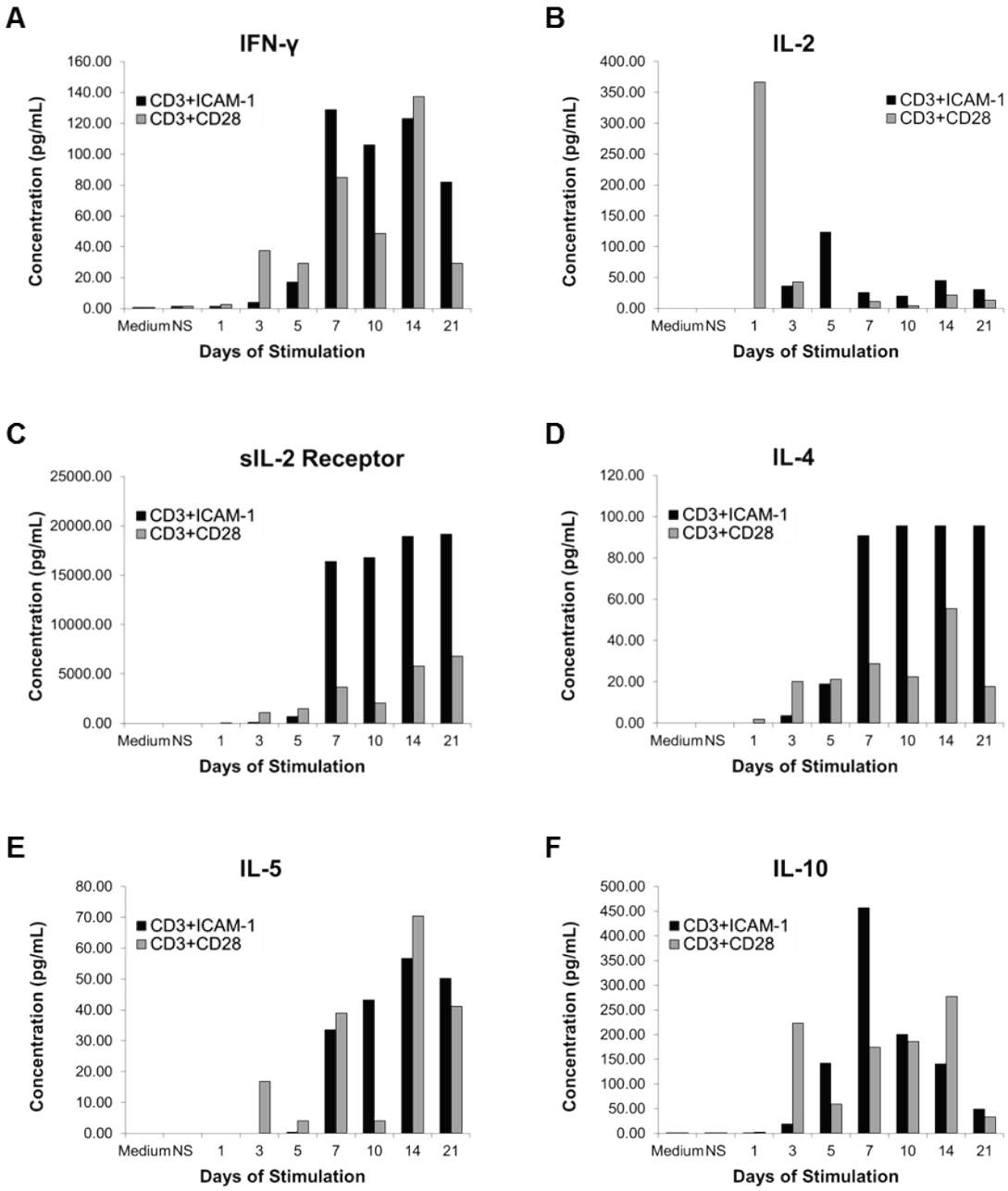
A
IFN-γ
Concentration (pg/mL)
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
B
IL-2
Concentration (pg/mL)
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
C
sIL-2 Receptor
Concentration (pg/mL)
25000.00
20000.00
15000.00
10000.00
5000.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
D
IL-4
Concentration (pg/mL)
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
E
IL-5
Concentration (pg/mL)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
F
IL-10
Concentration (pg/mL)
500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation

Figure 5.4. Luminex "Cytokine II" Panel. Cell culture supernates were collected and analyzed by Luminex for the following analytes: A) IFN-α, B) IL-7, C) IL-12, D) IL-13, E) IL-15, F) IL-17. Medium = complete RPMI medium, NS = nonstimulated. Data are the mean of duplicate samples from one experiment.

Figure 5.4

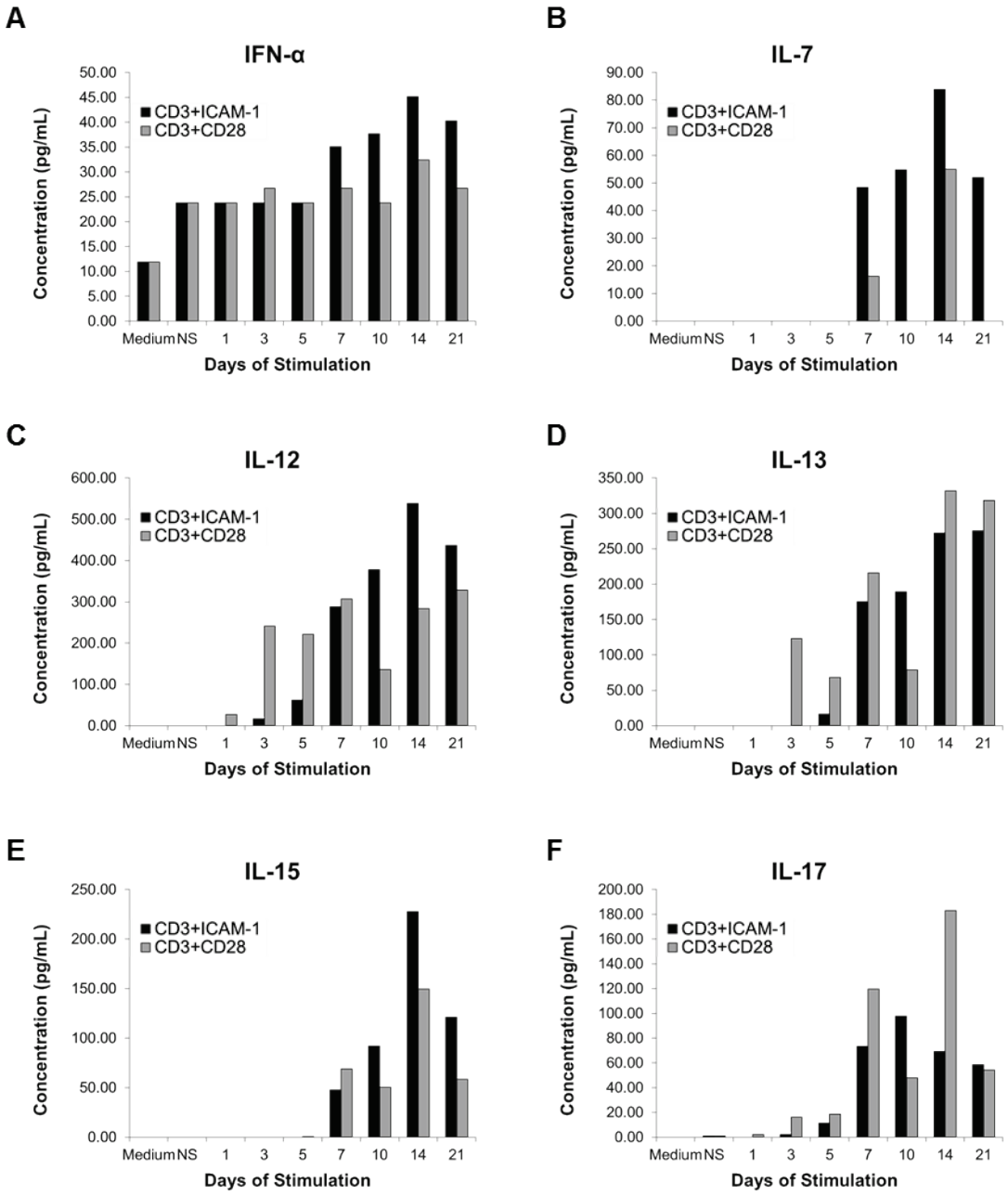
A
IFN-α
Concentration (pg/mL)
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
B
IL-7
Concentration (pg/mL)
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
C
IL-12
Concentration (pg/mL)
600.00
500.00
400.00
300.00
200.00
100.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
D
IL-13
Concentration (pg/mL)
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
E
IL-15
Concentration (pg/mL)
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
F
IL-17
Concentration (pg/mL)
200.00
180.00
160.00
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation

Figure 5.5. Luminex "Chemokine" Panel. Cell culture supernates were collected and analyzed by Luminex for the following analytes: A) Eotaxin, B) IP-10, C) MCP-1, D) MIG, E) MIP-1α, F) MIP-1β, G) RANTES. Medium = complete RPMI medium, NS = nonstimulated. Data are the mean of duplicate samples from one experiment.

Figure 5.5

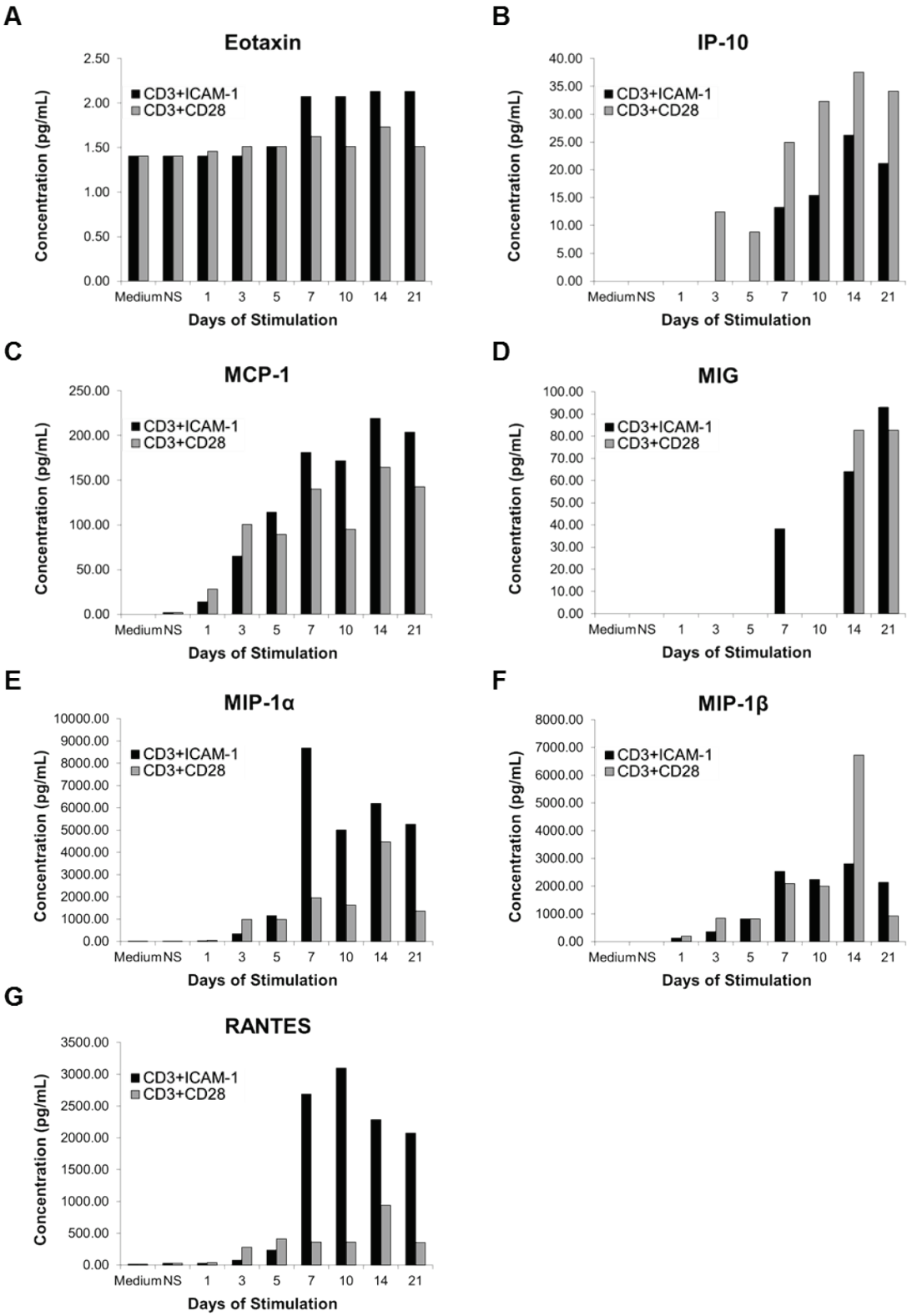
A
Eotaxin
Concentration (pg/mL)
2.50
2.00
1.50
1.00
0.50
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
B
IP-10
Concentration (pg/mL)
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
C
MCP-1
Concentration (pg/mL)
250.00
200.00
150.00
100.00
50.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
D
MIG
Concentration (pg/mL)
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
E
MIP-1α
Concentration (pg/mL)
10000.00
9000.00
8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
F
MIP-1β
Concentration (pg/mL)
8000.00
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation
G
RANTES
Concentration (pg/mL)
3500.00
3000.00
2500.00
2000.00
1500.00
1000.00
500.00
0.00
CD3+ICAM-1
CD3+CD28
Medium NS 1 3 5 7 10 14 21
Days of Stimulation

Table 5.2. Summary of Luminex results indicating the stimulation treatment that favored production of each cytokine and chemokine.

Table 5.2

Higher Concentrations with CD3+ICAM-1	Higher Concentrations with CD3+CD28	Similar Concentrations with Both Stimuli	Low Concentrations with Both Stimuli (< 50 pg/mL)
IL-1R antagonist	TNF-α	GM-CSF	IL-6
IL-8/CXCL8	IL-2	IL-1β	IFN-α
sIL-2Rα	IL-17	IFN-γ	Eotaxin/CCL11
IL-4	MIP-1β/CCL4	IL-5	IP-10/CXCL10
IL-10		IL-7	
IL-12		IL-13	
IL-15		MCP-1/CCL2	
MIP-1α/CCL3		MIG/CXCL9	
RANTES/CCL5			

Figure 5.6. Kinase Substrate Array. Naïve CD4+ T cells were stimulated for 4.5 days through either CD3+ICAM-1 or CD3+CD28. Cell lysates were prepared and a Kinase Substrate Array experiment was performed as described in the Materials and Methods. Densitometry was used to assign a score to each spot by calculating the % Difference from Background using the equation: [(Densitometry value of spot – Average densitometry value for negative control spots)/ Average densitometry value for negative control spots] x 100%. Negative control spots used in calculating the % Difference from Background score are outlined in blue. Duplicate spots that both had a score equal to or greater than 3.0 are outlined in red and listed in **Table 5.3**. Data were collected from one experiment.

Figure 5.6

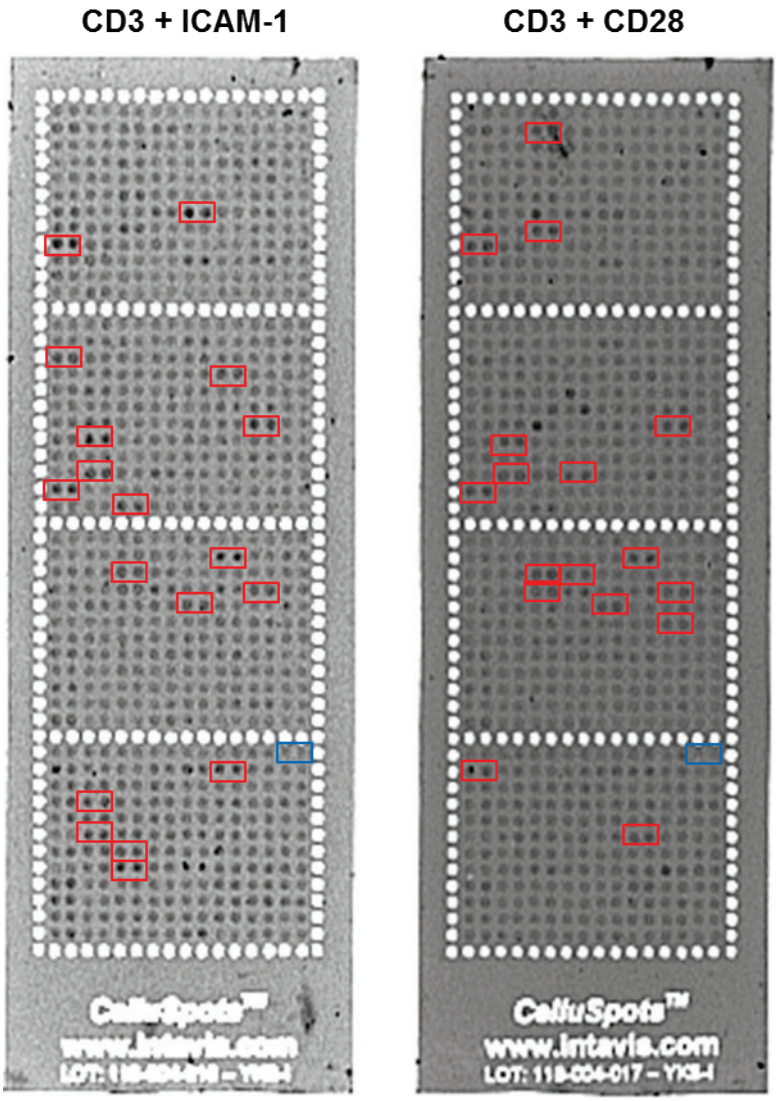
CD3 + ICAM-1
CD3 + CD28
CellaSpots™
www.Interviscom
CellaSpots™
www.Interviscom

Table 5.3. The kinases that are expected to phosphorylate the spots identified in **Figure 5.6** are listed along with their corresponding full names and common alternative names. The OMIM (Online Mendelian Inheritance in Man) website (www.omim.org) was referenced order to obtain the relevant information in the table.

Table 5.3

Kinase	Description/Alternative Names
CD3+ICAM-1 Array	
InsR	Insulin receptor
Met	HGFR (hepatocyte growth factor receptor)
Csk	cytoplasmic tyrosine kinase; c-Src tyrosine kinase
PDGFR group	Platelet-derived growth factor receptor group
PKCα	Protein kinase C, alpha
PKCε	Protein kinase C, epsilon
CD3+CD28 Array	
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2; HER-2/neu
FAK	Focal adhesion kinase; PTK2 (protein-tyrosine kinase)
Lck	Lymphocyte-specific protein-tyrosine kinase; p56
Src group	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene group
Both Arrays	
EGFR	Epidermal growth factor receptor; ErbB1; HER1
FLT3	Fms-related tyrosine kinase 3; STK1 (stem cell tyrosine kinase 1)
IGF1R	Insulin-like growth factor 1 receptor
Lyn	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
PDGFRβ	Platelet-derived growth factor receptor, beta
Src	v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene
Syk	Protein-tyrosine kinase Syk; spleen tyrosine kinase

DISCUSSION

The results shown in this chapter suggest that there are differences in both signaling and functional outcome after ligation of ICAM-1 versus CD28 during naïve CD4+ T cell activation. Activation occurs more rapidly after costimulation through CD28 than through ICAM-1 (**Fig. 5.1**). One possible explanation for this result is the higher expression of CD28 on naïve CD4+ T cells. It is possible that ICAM-1 expression must be upregulated before signaling using anti-ICAM-1 can occur effectively. Another possibility would be if CD28 delivered a stronger costimulatory signal leading to more rapid activation, or if cytokines that promoted activation were secreted earlier after CD28 costimulation.

There were differences in cytokine and chemokine secretion between CD3+ICAM-1 and CD3+CD28 stimulation detected in the Luminex assay (**Fig. 5.2-5.5**). Usually the peak cytokine concentration occurs between Days 7-21 of stimulation, but in some cases, cytokine secretion occurs sooner with CD3+CD28 which corresponds with the faster activation shown in **Figure 5.1**. In this Luminex experiment, we found that both ICAM-1 and CD28 could produce both T_{H1} and T_{H2} cytokines. These results differ from those previously obtained from our lab using ELISA, which showed that ICAM-1 could induce IFN-γ and IL-2, but not IL-5, while CD28 could induce secretion of all three cytokines (8). However, there are differences between the methodology of these experiments in both the type of cell populations studied (total T cells previously and naïve CD4+ T cells in this chapter) and the timing of analysis (24 hours of stimulation previously and 1-21 days in this experiment). Also, it should be noted that this Luminex experiment studied cytokine secretion using cells from one subject, and is therefore preliminary.

Although preliminary, some of the differences in cytokine/chemokine secretion were especially interesting to us. One such result was that higher levels of MIP-1α (CCL3) and RANTES (CCL5) were secreted after costimulation through ICAM-1, while higher levels of MIP-1β (CCL4) were secreted after costimulation through CD28 (**Fig. 5.5**). These data correspond with a gene array that former graduate student Jake Kohlmeier performed in which he found higher MIP-1α and RANTES (but not MIP-1β) mRNA expression after costimulation through ICAM-1 when compared with costimulation through CD28 (24) and a previously published study that showed RANTES production after stimulation of ICAM-1 on human bronchial epithelial cells (25). MIP-1α can bind to the chemokine receptors CCR1 and CCR5, while RANTES can bind to CCR1, CCR3, CCR5, Duffy, and D6, and MIP-1β can bind to CCR5, CCR8, and D6, and is an antagonist for CCR1 (22). A recent paper showed that TGF-β-induced mouse T_{reg} cells could secrete MIP-1α and MIP-1β, and these chemokines could enhance the *in vitro* migration of other T_{reg} cells as well as suppress autoimmune gastritis *in vivo*. In contrast, effector T cells could secrete RANTES, and this chemokine could enhance the *in vitro* migration of other effector cells (26). So even though MIP-1α, MIP-1β, and RANTES share the receptors CCR1 and CCR5, they can exert different effects on the immune system.

Another interesting result in the Luminex experiment was the high secretion of soluble IL-2Rα (sIL-2Rα/soluble CD25) after CD3+ICAM-1 stimulation. This was interesting since we observe a population of $Foxp3^{hi}CD25+$ T_{reg} cells after costimulation through ICAM-1 (Chapter 2, (27)). However, we cannot determine whether the sIL-2Rα detected had been cleaved from the surface of the T_{reg} cells in our culture or another cell subset that expresses IL-2Rα/CD25. The precise function for sIL-2Rα is unknown, although it is detected in the serum of patients with some cancers, infections, autoimmune diseases, and inflammatory diseases. Two recent papers

proposed opposing roles for sIL-2Rα released by T_{reg} cells in mediating suppression. One group hypothesized that T_{reg} cells may release sIL-2Rα to bind IL-2 and prevent the IL-2 from being able to help activate effector T cells. They showed that recombinant sIL-2Rα could decrease anti-CD3 plus IL-2 induced T cell proliferation *in vitro* by 50% (28). In contrast, another group found that rather than blocking the function of IL-2, sIL-2Rα bound to IL-2 could enhance the function of IL-2 by increasing Stat5 phosphorylation and promoting differentiation to T_{reg} cells. They found that adding sIL-2Rα to anti-CD3 plus IL-2 stimulated T cells *in vitro* could increase proliferation (29).

We also detected differences in the kinases activated after costimulation through ICAM-1 versus through CD28 (**Fig. 5.6**). The only day studied was Day 4.5 of stimulation, so it is not known whether there are kinetic differences in kinase activation or if there are truly different kinases activated due to different signaling cascades. Some of the kinases identified were growth factor receptors with tyrosine kinase activity (*e.g.* PDGFRβ), while some were proto-oncogenes involved in activation after TCR signaling (*e.g.* Lck) or in cell proliferation (*e.g.* Src).

Because we analyzed cytokine/chemokine secretion and kinase activation from the entire population of stimulated naïve CD4+ T cells, we cannot determine which cell population secreted each particular cytokine and chemokine or expressed each activated kinase. It is possible that differences found between CD3+ICAM-1 and CD3+CD28 stimulated samples were related to the presence of T_{reg} cells that differentiated in the CD3+ICAM-1 stimulated cultures, either because the cytokines/chemokines or activated kinases promoted T_{reg} differentiation or because the T_{reg} cells themselves expressed these cytokines/chemokines or kinases.

With a variety of costimulatory molecules expressed on the T cell surface and the corresponding ligands expressed on APCs, the question arises as to why more than one type of costimulatory molecule is utilized. Does stimulating through different costimulatory molecules lead to different functional outcomes? If different functional outcomes exist, are they due to differences in signaling, or instead due to other factors? One possibility is that different costimulatory molecules have the same function, but act on different subsets of T cells or at different stages of the immune response to produce different outcomes. A second possibility is that the signaling pathways used by different costimulatory molecules truly differ and therefore lead to different outcomes. These two possibilities are not necessarily mutually exclusive.

Our data using our *in vitro* cell differentiation model suggest that differentiation outcomes can differ between costimulatory molecules. For example, when comparing costimulation of naïve CD4+ T cells between ICAM-1 and CD28, costimulation though either molecule can lead to proliferation, protection from apoptosis, and differentiation to effector and memory cells (10), but ICAM-1 can and CD28 cannot lead to differentiation of $Foxp3^{hi}$ regulatory T cells (27). **Figures 5.2-5.5** in this chapter demonstrate further that cytokine production can vary between stimuli. Our model also suggests that costimulatory molecules can use different signaling pathways. Supporting evidence for this is shown in **Figure 5.6** in this chapter.

A possibility not addressed by our data is whether costimulatory molecules act on different subsets of cells. This seems likely in the context of the many T cell and APC populations involved in different stages of the immune response. Although the naïve CD4+ T cells population is generally thought of as primarily a homogeneous population, new evidence suggests that subpopulations may exist within the naïve population. For example, Bendall and

colleagues used single-cell “mass cytometry” to group naïve CD4+ T cells into 16 distinct subsets based on varied expression of 13 cell-surface antigens (30). However, it is not known whether stimulation of these subsets by ICAM-1 or CD28 would lead to different functional outcomes of these newly proposed subsets of naïve cells.

CHAPTER 5 ACKNOWLEDGEMENTS

Dr. Abby Dotson and I collaborated on the Luminex and Kinase Substrate Array experiments, and some of the results shown in this chapter also appear in her dissertation. I would like to thank Dr. Marcia Chan and Nicole Gigliotti at Children's Mercy Hospital who provided equipment, technical advice, and assistance for the Luminex and Kinase Substrate Array experiments. Previous experiments in our lab comparing stimulation of naïve CD4+ T cells through ICAM-1 and through CD28 were performed by former graduate student Dr. Jake Kohlmeier and appear in his dissertation and the cited references.

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CHAPTER 6

CONCLUSIONS

DISSERTATION CONCLUSIONS

Naïve CD4+ T cells that are activated through a TCR antigenic signal plus a costimulatory signal can proliferate and differentiate into a variety of T cell subsets. These subsets include effector T cells (*e.g.* T_{H1}, T_{H2} cells), memory T cells, and T_{reg} cells. A variety of signals can influence the eventual cell fate of the differentiating naïve CD4+ T cell. First, cues in the microenvironment such as cytokines, chemokines, growth factors, and costimulatory ligands expressed by APCs can direct T cell phenotype and function (1). Second, new evidence suggests that naïve CD4+ T cells can be subdivided based on surface molecule expression (2). Differential expression of costimulatory ligands or cytokine receptors by the naïve T cell would be expected to impact differentiation potential. In this scenario, the same microenvironment could yield distinct differentiation outcomes due to naïve T cell-specific differences. In this dissertation, we presented data that 1) further described the role of ICAM-1 in the activation and differentiation of naïve T cells and 2) supported the concept that cell fate can be influenced by the costimulatory signal.

A previously unknown role for ICAM-1 in the generation of inducible regulatory T (iT_{reg}) cells was described in Chapter 2. Stimulation of human naïve CD4+ T cells through CD3+ICAM-1 induced a population of cells with a standard T_{reg} phenotype: $Foxp3^{hi}$CD25+CTLA-4+$CD127^{lo}$ (**Fig. 2.2**). In contrast, a population of $Foxp3^{hi}$ cells was not detected after stimulation through CD3+CD28. IL-2 signaling was necessary for T_{reg} induction *in vitro* after costimulation through ICAM-1 (**Fig. 2.6**). We also verified that the T_{reg} cells induced after ICAM-1 costimulation could function to inhibit responder cell proliferation in an *in vitro* suppression assay (**Fig. 2.9**).

In Chapter 3, we studied the *in vitro* immune responses of naïve CD4+ T cells from older individuals, aged 65 years and older. In contrast to the results obtained with naive CD4+ T cells from younger individuals, naïve CD4+ T cells from older individuals did not typically differentiate into a subset of T_{reg} cells after costimulation through ICAM-1 (**Fig. 3.5**). However, the percentage of $Foxp3^{hi}$ cells did increase in one preliminary experiment if exogenous TGF-β1 and IL-2 were added to the stimulation treatment (**Fig. 3.6**). These preliminary data suggest that naïve CD4+ T cells from older individuals retained the ability to differentiate to T_{reg} cells, but this differentiation was not associated with ICAM-1 signaling. However, aging did not appear to affect the ability of costimulation through ICAM-1 to generate effector and memory T cells (**Fig. 3.4**, Abby Dotson's dissertation). These *in vitro* data suggest a skewing of differentiation toward effector and memory subsets and away from the T_{reg} subset after naïve CD4+ T cells from older individuals are costimulated through ICAM-1.

Chapter 4 examined the effect of costimulation through ICAM-1 expressed on mouse T cells, both *in vitro* and *in vivo*. When mouse CD4+ T cells were stimulated with antibodies against CD3 and ICAM-1, we did not detect cells with a T_{reg} phenotype or with suppressive function unless exogenous TGF-β1 plus IL-2 were included in the culture conditions (**Fig. 4.2, 4.3**). However, we also did not observe typical signs of costimulation such as increased proliferation, which might indicate that the antibodies that were used to attempt to stimulate ICAM-1 were not effective. Another possibility is that the mouse ICAM-1 cytoplasmic domain might lack specific motifs necessary for certain signal transduction pathways. However, our *in vivo* studies that assessed CD8+ T cell cytokine production, cytotoxic ability, and memory T cell differentiation during VSV infection suggest that mouse ICAM-1 can function as a signaling molecule; although ICAM-1 was not required for the parameters we evaluated (**Fig. 4.5, 4.8**).

Interestingly, ICAM-1$^{-/-}$ mice had slightly elevated immune responses compared to wild-type controls. ICAM-1$^{-/-}$CD28$^{-/-}$ mice had decreased acute responses compared to ICAM-1$^{-/-}$ mice and wild-type controls, but slightly increased memory responses compared to wild-type controls (**Fig. 4.4-4.8**). Together, these data demonstrate that a deficiency in both of these important costimulatory molecules does not completely prevent CD8+ T cell activation, differentiation, and function, and may even suggest opposing roles for ICAM-1 and CD28 during the immune response to VSV.

Finally, in Chapter 5, we provided supporting evidence for the concept that differential costimulatory signals can help direct T cell differentiation outcome. Activation and proliferation was initiated more rapidly after costimulation through CD28 than through ICAM-1 (**Fig. 5.1**). Data from a preliminary Luminex experiment demonstrated differences in cytokine and chemokine secretion after costimulation through ICAM-1 versus CD28 (**Fig. 5.2-2.5**), while a preliminary Kinase Array showed differences in kinase activation (**Fig. 5.6**). These results indicate distinct signaling profiles and functional outcomes after costimulation through ICAM-1 compared to costimulation through CD28.

Our Chapter 2 results demonstrating that a subset of naïve CD4+ T cells costimulated through ICAM-1 can differentiate to a population of Foxp3^{hi} T_{reg} cells differ from our results in Chapter 3 and Chapter 4 where differentiation to a T_{reg} phenotype does not appear to occur after costimulation of naïve CD4+ T cells from older individuals or CD4+ T cells from mice. It is known that IL-2 is essential for T_{reg} differentiation (3), and this IL-2 requirement was confirmed in our system as well (**Fig. 2.6**). We did not assess IL-2 secretion when naïve CD4+ T cells were obtained from older individuals or when CD4+ T cells were obtained from mice, so we do not know whether IL-2 production was normal or impaired. In the case of the mouse CD4+ T cells,

two other possibilities for a lack of T_{reg} induction are mentioned above. It is worth noting that cells from an older individual (**Fig. 3.6**) or from mice (**Fig. 4.2**) that were stimulated in the presence of exogenous IL-2 plus TGF-β1 did display a $Foxp3^{hi}$ T_{reg} phenotype. It might be useful to determine if there are any phenotypic or functional differences between cytokine-induced T_{reg} cells that differentiate in the presence of CD3+ICAM-1 stimulation versus CD3+CD28 stimulation.

Another interesting observation in Chapter 4 was that the immune response to VSV was lowered in ICAM-$1^{-/-}$$CD28^{-/-}$ mice, but slightly heightened in ICAM-$1^{-/-}$ mice. Because we no longer had a colony of $CD28^{-/-}$ mice at the time these experiments were performed, we do not know whether the response of the ICAM-$1^{-/-}$$CD28^{-/-}$ mice would have been intermediate to the ICAM-$1^{-/-}$ mice and $CD28^{-/-}$ mice, or lower than the $CD28^{-/-}$ mice. Also, because we did not test for the presence of T_{reg} cells during the VSV experiments, we do not know whether the slightly increased response in the ICAM-$1^{-/-}$ mice was due to a T_{reg} defect. Another group found a similarly increased immune response in ICAM-$1^{-/-}$ mice infected with *M. tuberculosis*, and attributed this result to decreased T_{reg} numbers and impaired T_{reg} signaling (4). However, as mentioned in Chapter 4, knocking out specific costimulatory molecules can produce vastly different results depending upon the disease model studied.

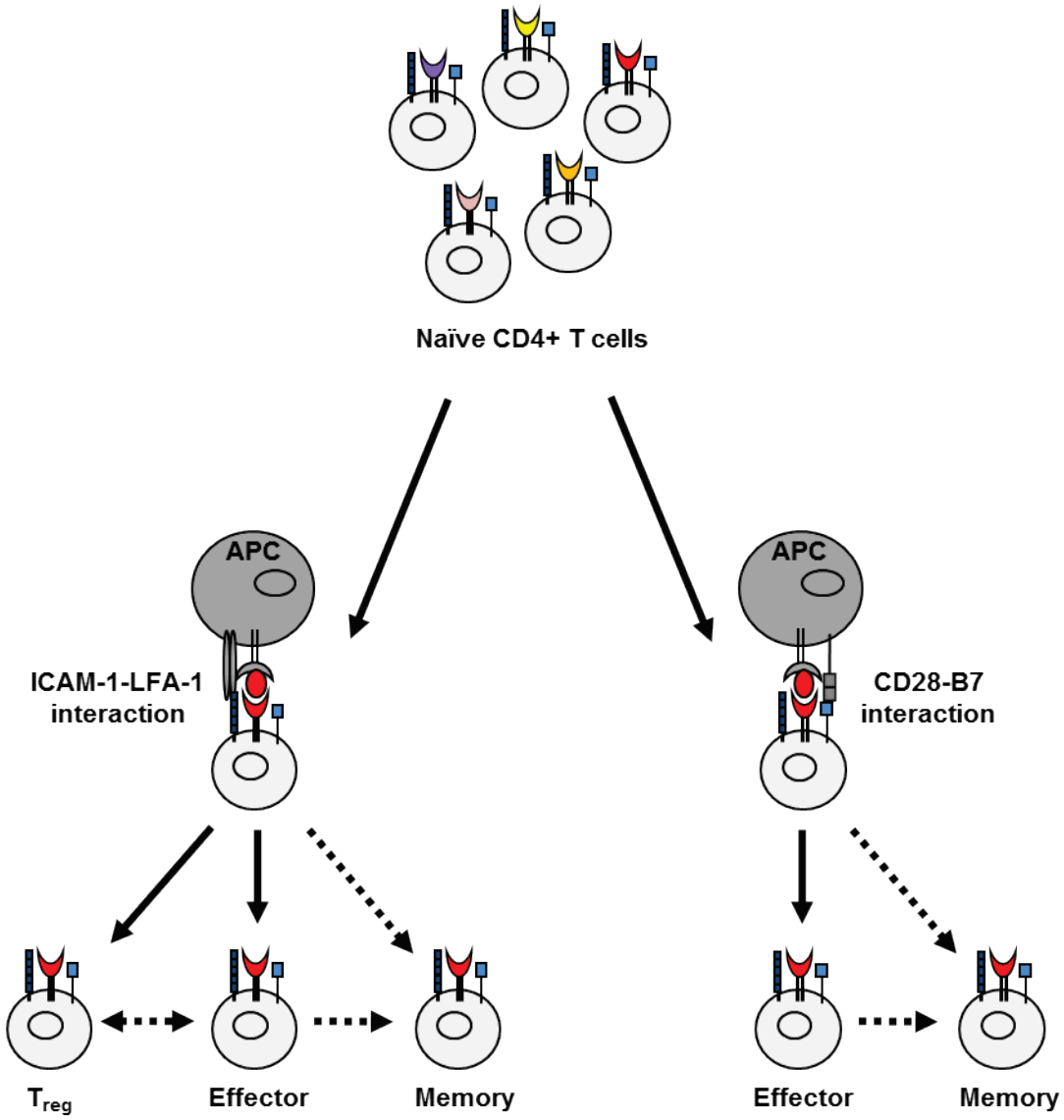
Some of the results of costimulation through ICAM-1 were the same as those through CD28, while others were different. For example, our lab has previously published that naïve CD4+ T cells costimulated through either molecule can lead to proliferation, protection from apoptosis, secretion of IL-2, and differentiation to effector and memory subsets (5). However, as reported here, a subset of T_{reg} cells were also generated after costimulation through ICAM-1, but not CD28 (6). CD8+ T cell responses to VSV were generally slightly increased in mice lacking

ICAM-1, while acute CD8+ T cell responses generally decreased in mice lacking both ICAM-1 and CD28. In addition, differential cytokine/chemokine and kinase activation profiles were observed in preliminary experiments comparing costimulation through ICAM-1 or CD28. Together, these results might suggest that some of the signaling pathways utilized by ICAM-1 and CD28 are shared, leading to some similar outcomes, while other pathways are distinct, leading to some unique outcomes.

Based on our results, we propose a model whereby the costimulatory signal received by the naïve CD4+ T cell can influence differentiation outcome (**Fig. 6.1**). We do not know whether the observed effect of costimulation on differentiation is due to a direct effect of ICAM-1 signaling versus CD28 signaling (*e.g.* determined by differential kinases activated during signaling), or rather is an indirect effect (*e.g.* due to differential cytokine secretion that then impacts differentiation). In addition, we do not know the timing or location that ICAM-1 costimulation might be especially important during *in vivo* immune responses. ICAM-1 will interact strongly with its ligands (*i.e.* β2 integrins) when these ligands are in an active conformation (7). This suggests that costimulation through ICAM-1 would occur when cognate antigen is presented by an activated APC. From our *in vitro* results, we might also speculate that the timing of activation and differentiation might be slower after costimulation through ICAM-1 compared to CD28, and that ICAM-1 might both initiate effector immune responses and assist in T_{reg}-mediated tolerance.

Figure 6.1. The outcome of differentiation can be influenced by costimulation. Naïve CD4+ T cells encounter cognate antigen presented in the context of MHC Class II molecules on APCs along with costimulatory signals. The *in vitro* data presented in this Dissertation suggest that the differentiation possibilities differ when the dominant costimulatory signal is received through ICAM-1 compared to CD28.

Figure 6.1


Naïve CD4+ T cells
APC
APC
ICAM-1-LFA-1
interaction
CD28-B7
interaction
T_{reg}
Effector
Memory
Effector
Memory

FUTURE DIRECTIONS

There are many interesting paths that this research could follow in the future. As one avenue of investigation, it would be important to identify which natural ligands of ICAM-1 can provide a costimulatory signal (*e.g.* LFA-1, Mac-1), or if there is a difference between a signal received from LFA-1 versus a signal received from Mac-1. This study might begin by using recombinant proteins instead of stimulating antibodies, or by stimulating using APCs and blocking the molecules of interest. It would also be important to assess how the ligands (i.e. β2 integrins) might influence ICAM-1 signaling when the β2 integrins are in an active versus an inactive conformation. Work from another group indicates that when LFA-1 or Mac-1 expressed on mouse DCs is in an active conformation, DC-T interaction times increase but T cell proliferation actually decreases (8, 9).

It would also be valuable to determine what specific signals initiated by ICAM-1 ligation might lead to Foxp3 induction and T_{reg} differentiation, and how they might differ from signals from CD28 ligation that did not lead to T_{reg} differentiation. We do not know whether this is a direct effect of ICAM-1 signaling, or is an indirect effect, perhaps mediated by cytokines produced after costimulation through ICAM-1. Some possible cytokines produced after ICAM-1 costimulation that are implicated in T_{reg} induction are IL-2, TGF-β1, and IL-10. Our data already indicates that IL-2 is necessary for T_{reg} induction (**Fig. 2.6**). Windish *et al.* identified that the TGF-β Smad pathway was impaired in ICAM-$1^{-/-}$ mice, but speculated that this was due to a lack of signaling through LFA-1 (4). Our studies indicate that signaling through ICAM-1 itself on the T cell is important for T_{reg} differentiation. However, it would be interesting to determine if ICAM-1 signaling activates components of the Smad pathway to facilitate Foxp3 induction.

The preliminary Luminex experiment shown in Chapter 5 provided several possibilities for further study. We found that naïve CD4+ T cells costimulated through ICAM-1 produced higher concentrations of the chemokines RANTES and MIP-1α than was detected after costimulation through CD28 (**Fig. 5.5**). This corresponds with data from a gene array previously performed by Jake Kohlmeier in our lab (10). One of our next objectives is to determine which cell type is producing these chemokines, which cell types can respond to these chemokines, and what type of response these cells have (*e.g.* migration, differentiation). Since high levels of soluble IL-2Rα (soluble CD25) were detected in supernates from naïve CD4+ T cells costimulated through ICAM-1, but not CD28 (**Fig. 5.3**), it would also be interesting to determine which cell type is the source of this soluble cytokine receptor. Since T_{reg} cells express high levels of surface CD25, and T_{reg} cells were found in cultures of naïve CD4+ T cells costimulated through ICAM-1 but not CD28, it is tempting to speculate that the T_{reg} cells might be the source of the soluble IL-2Rα. If this were the case, it would be valuable to determine what effect, if any, the soluble IL-2Rα has on the function of other leukocytes.

We demonstrated that the ICAM-1-induced T_{reg} cells can suppress the proliferation of responder T cells (**Fig. 2.9**), but we did not identify the specific mechanism of suppression. Potential mechanisms may include perforin, granzyme B, TGF-β, IL-10, or acting an "IL-2 sink" (11). Additional suppression assays could be performed while inhibiting the molecules of interest. Additional flow cytometry staining could be used to detect possible perforin and granzyme B expression in T_{reg} cells.

Understanding the mechanisms by which ICAM-1 costimulation leads to activation and differentiation might also possibly assist in the development of immune-targeted therapies. A long-term goal of this research is to determine if ICAM-1 induced T_{reg} cells might be viable for

use in adoptive regulatory T cell therapy for diseases such as autoimmunity or transplantation tolerance (12, 13). The research presented in this Dissertation promotes the concept that ICAM-1 expressed on the naïve T cell surface has distinct roles in both immunity and tolerance and should continue to be studied for possible future clinical applications.

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ABBREVIATIONS

APC	Antigen Presenting Cell
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CTL	Cytotoxic T Lymphocyte
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
ELISA	Enzyme-Linked Immunosorbent Assay
FOXP3	Forkhead Box P3
GALT	Gut-Associated Lymphoid Tissue
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HSC	Hematopoietic Stem Cell
ICAM-1	Intercellular Adhesion Molecule-1
Ig	Immunoglobulin
IL	Interleukin
LAP	Latency-Associated Peptide
LFA-1	Leukocyte Function-associated Antigen-1
LPAM-1	Lymphocyte Peyer's patch Adhesion Molecule-1
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphocyte Reaction
MS	Multiple Sclerosis
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PHA	Phytohemagglutanin
RBC	Red Blood Cell
SEM	Standard Error of the Mean
TCR	T Cell Receptor
TGF-β1	Transforming Growth Factor-β1
T_C	T cytotoxic
T_H	T helper
T_{reg}	T regulatory
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor-α
TNFR	Tumor Necrosis Factor Receptor
VLA-4	Very Late Antigen-4
VSV	Vesicular Stomatitis Virus
WBC	White Blood Cell

APPENDIX

SELECTED PROTOCOLS

Protocol 1: Purification of PBMCs from Peripheral Blood

1. Warm tissue culture (TC) PBS (containing 2% FBS, 1% Penicillin/Streptomycin) at 37° C

2. After drawing blood:

    -eject blood into appropriately sized bottle or tube depending upon the volume of blood

    -Dilute 1 part TC PBS : 2 part blood

    -Gently swirl bottle

3. Determine number of 50 mL tubes to use

    -can use 40 mL [blood + TC PBS] per 50 mL tube

4. Pipet 9 mL Ficoll-Paque PLUS into each tube

5. **Overlay** blood over Ficoll

    -add 25 mL blood to all tubes, then add the remaining 15 mL blood

    -place pipet tip just above Ficoll and expel blood very slowly

    -when expelling blood, keep tip above blood and make 2 points of entry

6. Centrifuge 30 minutes, room temperature, 1800 rpm (Beckman Allegra™ 6R Centrifuge), **No brake**

7. After blood has spun:

    -Place four new 50 mL tubes in hood

        -In the spun tubes, aspirate most of the top (plasma) layer (leave about 10 mL above the PBMC layer)

    -Place the pipet tip just above the PBMC layer and move pipet slowly in circle while pipetting up PBMC layer (will be about 10 mL)

-Don’t pipet up any RBCs

-Pipet PBMCs into the 4 new tubes in approximately equal volumes

8. Fast spins:

-Wash 2x after adding approximately 20 mL TC PBS to each tube

-Centrifuge at 2,000 rpm, 10 minutes, room temperature

-after the first wash, combine PBMCs into 2 tubes (40 mL TC PBS per tube)

-after the second wash, combine PBMCs into 1 tube (40 mL TC PBS per tube)

9. Slow spins:

-Wash again (the number of slow spin washes depends upon how much blood is drawn and how cloudy the sample is, which indicates how many platelets remain)

-Centrifuge at 800 rpm, 10 minutes, room temperature

10. Determine PBMC count

11. When applicable, proceed to the appropriate magnetic cell separation procedure (*e.g.* StemSep Human Naïve CD4+ T Cell Enrichment Kit)

Protocol 2: Purification of Total T cells from Tonsil

1. Prepare Sheep Red Blood Cells (SRBCs)

-Dissolve 0.5 g AET in 12.5 mL ddH_2O (this amount is appropriate for 15 mL SRBCs)

-pH AET solution to pH = 9.0 with NaOH

-filter sterilize AET solution through 0.2 μm syringe filter

-Remove 15 mL SRBCs from bottle by pipetting

-Eject the SRBCs slowing into a tube

-Wash the SRBCs (15 mL) with 25 mL cold TC PBS (containing 1% Penicillin/Streptomycin)

-centrifuge at 4°C, 2000 rpm (Beckman Allegra™ 6R Centrifuge), 10 minutes

-should yield approximately a 5 mL pellet of SRBCs

-Add 12 mL AET solution to SRBC pellet, resuspend

-Place in 37°C water bath for 20 minutes

-Add 25 mL cold TC PBS

-Wash 3x (or until supernate is clear) at 30 mL total volume

-Centrifuge each time at 2000 rpm, 10 minutes, 4°C, aspirate supernate

-Resuspend RBCs in TC PBS (add 25 mL to the 5 mL pellet)

2. Purify WBCs from Tonsils

-Place 5 50 mL tubes in hood (4 are for Tonsil cells and 1 is for Ficoll)

-Aspirate any extra fluid in the tonsil tube (Flame opening of tonsil tube before and after opening)

-Place 1 medium (20 mL) Petri dish in hood

-Open foil-wrapped tools in hood, put strainer in Petri dish

-Dip tonsil into 70% ethanol solution for 5 seconds, then dip tonsil into complete RPMI 1640 medium to wash

-Place tonsils into strainer

-To remove cells from the tonsil:

-Add approximately 45 mL warm TC PBS (containing 2% FBS, 1% Penicillin/Streptomycin) to strainer

-Use forceps and scissors to cut tonsil into small pieces

-Lift strainer

-Use a 10 mL pipet to wash the tonsil 3x with the cell solution

-Pipet cell solution into the 4 tubes

-Repeat this process 2x, but also use the pestle to gently mash the tonsil tissue

-Should end with approximately 30 mL cell solution per tube

-Ficoll underlay:

-Place approximately 45 mL Ficoll in the 5^{th} tube in the hood

-Add Ficoll to the 4 cell solution tubes at a 1:3 Ficoll:WBC solution ratio

-Place pipet tip at bottom of tube and slowly add Ficoll

-centrifuge 1800 rpm, 30 minutes, room temperature, minimum brake

-Separating the WBCs:

-Place 2 50 mL tubes in hood

-When Ficoll and WBC solution tubes have finished spinning, aspirate some of the top (TC PBS) layer

-Use a 10 mL pipet to remove WBC layer (place tip at WBC/TC PBS interface)

-Don’t remove any RBCs

-Transfer WBCs to the 2 tubes in hood

-Add 20-25 mL warm TC PBS to tubes

-Wash WBCs: centrifuge 1800 rpm, room temperature, 8 minutes

-Aspirate supernates, combine pellets from the 2 tubes, add 20 mL warm TC PBS to each pellet, resuspend, and combine into 1 tube)

-Wash again, 1800 rpm, room temperature, 8 minutes

-Remove supernate from pelleted cells, resuspend in 40 mL warm TC PBS

3. Purify Total T Cells from the WBCs

-Determine WBC concentration using hemacytometer:

-Resuspend cells in the volume of warm TC PBS so the cells are at a concentration of 6.7×10^6 cells/mL

-Add up to 30 mL of WBC solution to new tubes

-Add warm TC PBS to each tube to bring total volume to 30 mL

-Add prepared SRBC solution at a 1:10 ratio

-An alternative to counting cells on hemacytometer:

-Add 25 mL TC PBS + FBS to 8 50 mL tubes

-Add 5 mL of cell solution to each tube

-Add 3 mL prepared SRBC solution per tube

-After the cells have been diluted to the proper concentration and the SRBCs have been added:

-Put tubes in the 37°C incubator for 10 minutes

-Centrifuge tubes 10 minutes, 4°C, 2000 rpm

-Place WBC-RBC tubes (with pellet) on ice for 30 minutes

-Place RPMI 1640 complete medium and the ACT bottle in the 37°C H_2O bath

-Carefully resuspend cells (still at 33 mL)

-Do a Ficoll underlay (at 1:3 Ficoll:Cell solution ratio)

-Add 10 mL Ficoll per tube by placing pipet tip at bottom of tube and slowly ejecting the Ficoll

-Centrifuge tubes 30 minutes, 1800 rpm, 4°C, minimum brake

-Return centrifuge temperature to 20°C after this spin

-Aspirate everything but the pellet (contains SRBCs bound to T cells)

-Aspirate the B cell layer first, then move aspirator pipet tip in circle on top of liquid

-Treat the pellets with ACT

-Add ACT at the same volume as you used Ficoll (10 mL per tube)

-Resuspend pellets and combine the solutions into 2 or more tubes

-Put tubes in 37°C water bath for approximately 7 minutes

-Remove the tubes after the solution has become darker red and less turbid

-Add enough warm TC PBS to go to 50 mL, mix by pipetting

-Centrifuge 8 minutes, room temperature, 1800 rpm

-Remove supernate and wash again 2x (add 20 mL warm TC PBS, resuspend, centrifuge 8 minutes, room temp, 1800 rpm)

-Remove supernate, resuspend cells in 50 mL complete RPMI 1640 medium

-Count T cells on hemacytometer and dilute the cell solution to $5x10^6$ cells/mL

-Pipet T cell solution into a large Petri dish and incubate overnight in 37°C incubator

Protocol 3: Plate-Bound Antibody Stimulation

1. Calculate the volume of each antibody (Ab) needed for 200 µL/well (for 96-well plate) of stimulating Ab solution. Antibodies are diluted in sterile PBS to make stimulating Ab solution.

2. Add 200 µL stimulating Ab solution per well and incubate in 37ºC incubator for 2 hours, or at 4ºC overnight.

3. Wash each well 3x with sterile PBS (with multi-channel pipet if doing multiple wells). With each wash, pipet out 200 µL of solution and discard, and then add 200 µL sterile PBS to the well. Take care not to let wells sit dry for too long during washes or before adding cells.

4. After washes, add cells to the 96-well plate at a concentration range of $1x10^6$ cells/ml to $2x10^6$ cells/ml by adding 200 µL of cells in complete RPMI 1640 medium per well (RPMI 1640 without L-glutamine + 10% FBS + 1% Pen/Strep + 1% L-glutamine). This is equal 200,000 to 400,000 cells per well. The number of cells to use depends upon the particular experiment being performed.

5. Incubate plate at 37ºC in incubator for the determined time to stimulate cells.

Protocol 4: Flow Cytometry Staining of Cell Surface Molecules

Staining Controls for Setting Flow Cytometer Parameters

-Unstained Cell Sample

-Cell Sample Single-Stained with FL1 (FITC) Ab only

-Cell Sample Single-Stained with FL2 (PE) Ab only

-Cell Sample Single-Stained with FL3 (PE-Cy5) Ab only

-Cell Samples stained with Abs plus with appropriate Isotype controls (if needed)

Staining Cell Surface Molecules

1. Prepare Staining Buffer (DPBS + 0.5% BSA), place in ice

2. Centrifuge cell samples (using Eppendorf Centrifuge 5417R at 4,000 rpm (1699 g) for 3 minutes at 4°C or using Labnet Spectrafuge 24D at 4,400 rpm (1700 g) for 3 minutes at room temperature), aspirate supernate

3. Resuspend cells in 100 µL Staining Buffer, Incubate on ice for 15 minutes

4. Centrifuge cell samples, resuspend in 100 µL Staining Buffer + Ab (1˚ Ab conjugated to fluorochrome)

Note: The proper amount of Ab to use varies, so each Ab should be titrated before use

5. Incubate cells in Ab for 20 minutes, on ice, in dark

Note: If the signal is too dim, the time for this incubation step can be increased

6. Wash: Add 500-900 µL Staining Buffer to sample, centrifuge cells, aspirate supernate (can wash again in Staining Buffer if needed)

7. Centrifuge cells, aspirate supernate, resuspend in 400 µL Staining Buffer

8. Pipet cell solution into flow cytometry tubes (If using the Accuri C6 flow cytometer, the cells can be resuspended in 200 µL volume in Step 7, and the cell solution can remain in Eppendorf tubes)

9. Keep on ice in the dark

10. Analyze on flow cytometer (or if waiting until the next day to analyze, add Paraformaldehyde solution to the cell solution so the final paraformaldehyde concentration is between 2-4%). Store at 4°C in the dark.

Notes: Add paraformaldehyde solution to a final concentration of between 2-4% paraformadehyde before flow cytometry analysis when using primary human cells or potentially infectious samples. Use caution when using paraformaldehyde due to its toxicity.

Protocol 5: Procedure for Removal of Antibody-Cytokine Complexes from Stimulated Cell Culture Supernates

-Use Protein G Sepharose 4 Fast Flow (Amersham/GE) so Rat IgG1 isotype can bind

-Protein G is at 75% in a 20% ethanol solution

1. Remove [30 µL Protein G solution x # samples] from stock inside hood
2. Centrifuge at 4°C, 1 minute, 11,000 rpm, aspirate supernate
3. Resuspend at 50% in 100 mM Tris pH 8.0
4. Centrifuge again, aspirate
5. Perform this wash 5x
6. Resuspend at 50% in 100 mM Tris pH 8.0
7. Add 30 µL solution to (# samples) tubes
8. Centrifuge, aspirate
9. Add 110 µL appropriate culture supernate to appropriate tube
10. Gently agitate tubes at 4°C for 60 minutes (shake 30 minutes, rotate 30 minutes)
11. Centrifuge, SAVE SUPERNATE
12. Centrifuge supernate collected in previous step, SAVE SUPERNATE
13. Supernates are ready for ELISA procedure
14. Save remaining supernates at -70°C

Protocol 6: CFSE Staining Protocol

-CFSE is from Molecular Probes (catalog # C1157), store at -20°C

-Make a stock solution of 5 mM CFSE in DMSO

-use sterile DMSO and add to the CFSE vial, then aliquot into several Eppendorf tubes

-Store stock solution at –20°C in the dark, with a desiccant present

1. Label **human cells with 2.5 µM CFSE** for 10 minutes at 37°C (in water bath) in the dark (tubes covered in foil) in serum-free medium (*e.g.* add 2.5 µL of 5 mM stock to 5 mL of cells in serum-free RPMI 1640 medium)
2. Label cells at a concentration of 2 million cells/mL

-The cells and CFSE should be mixed well so the CFSE stain will be uniform

3. Centrifuge cells at low speed for 5 minutes, remove supernate
4. Wash cells 2x in complete (with FBS) medium (*e.g.* complete RPMI 1640 medium)

Notes:

-If cell viability decreases, the staining is too bright, or the CFSE stock is newly made, might need to decrease the staining incubation time (*e.g.* to 7 minutes), decrease the CFSE concentration, or increase the cell concentration used.

-If staining **mouse cells**, it is better to use the CFSE at 1 µM final concentration and label the cells for 10 minutes at room temperature (instead of at 37°C).

-It is sometimes helpful to check the CFSE stain by flow cytometry before proceeding with plating the cells

Protocol 7: Suppression Assay using Human T cells

Week 1 (Day 0)

1. Isolate naïve CD4+ T cells

2. Stimulate cells using plate-bound anti-CD3 (clone OKT3, 1 µg/mL) and anti-CD54 (anti-ICAM-1, clone R6.5D6, 10 µg/mL)

3. Stimulate cells for 10 days

Week 2 (Day 10)

1. Isolate Total T cells from new blood sample from same donor = Responder T cells

2. Stain Responder cells with 2.5 µM CFSE

3. Isolate CD4+CD25+ cells from week 1 culture = Suppressor cells

4. Isolate CD4+CD25- cells from week 1 culture

5. Stain CD4+CD25+, CD4+CD25-, and a control group of total T cells with 2.5 µM PKH26 dye

6. Use the following culture conditions:

Group 1 (Control): PKH26-labeled Responder T cells + CFSE-labeled Responder T cells

Group 2: PKH26-labeled CD4+CD25+ cells + CFSE-labeled Responder T cells

Group 3: PKH26-labeled CD4+CD25- cells + CFSE-labeled Responder T cells

Each Group is cultured at Suppressor: Responder ratios of 1:1, 1:2, and 1:4

6. Stimulate cells with plate-bound anti-CD3 (1 µg/mL) + anti-CD28 (2 µg/mL) for 5 days

7. Perform flow cytometry

8. Determine the % Proliferation of CFSE-labeled Responder cells

Protocol 8: Purification of Mouse Splenocytes

1. Sterilely remove spleen and place in 4 mL complete RPMI 1640 (warmed to 37°C) in 14 mL tube

2. Open 70 µm mesh filter, place in small cell culture plate

3. Flame mouth of tube and pour spleen and medium into filter and plate

4. Alcohol flame scissors and forceps, Cut spleen into 4 pieces

5. Gentle mash spleen into mesh filter with sterile plunger from 1 cc syringe until the spleen tissue looks white

6. Use a 5 mL glass pipet to wash the cell solution over the mesh filter 3 times, Transfer all of the cell solution to a new 14 mL tube

7. Centrifuge tube at 70 speed (if using clinical centrifuge) for 5 minutes, Aspirate supernate

8. Resuspend quickly but gently in 5 mL **ACK** lysis buffer (room temperature), Incubate 5 minutes at room temperature

9. Add 5 mL complete RPMI 1640

10. Centrifuge at 70 speed for 5 minutes, Aspirate supernate

11. Resuspend in 5 mL complete RPMI 1640 (can pool spleens at this point if appropriate)

13. Count on hemacytometer (Make a 1:10 cell dilution in an Eppendorf tube for cell counting cells, *e.g.* 180 µL medium + 20 µL cells)

18. Proceed to next step of procedure (*e.g.* StemSep separation Protocol, MACS separation protocol, wash cells again, etc.)

Protocol 9: Removal of Dead Cells using Ficoll (or Lympholyte-M for Mouse Cells) for Small Volumes

1. Pool cells into Eppendorf tube (*e.g.* CD3 stimulated wells together into 1 tube, etc.)

2. Do a fast spin of cells in Eppendorf tubes

3. Resuspend cells in 500 µL complete RPMI 1640

4. Carefully layer cells over 500 µL Ficoll (or Lympholyte-M for mouse cells)

5. centrifuge at 4,000 rpm (1699 g) (using Eppendorf Centrifuge 5417R) at room temperature for 20 minutes

6. Carefully save cell layer above the Ficoll

7. Wash cells 2x in complete RPMI 1640 with fast spin (4,000 rpm) in Eppendorf tube

8. Perform cell count

9. Proceed to next step in experiment

Protocol 10: Suppression Assay using Mouse T cells

Week 1 (Day 0)

1. Isolate CD4+ T cells

2. Stimulate cells using plate-bound anti-CD3 (clone 500A2, 0.5 μg/mL) and anti-CD54 (anti-ICAM-1, clones KAT-1 or YN1/1.7.4, 10 μg/mL) or anti-CD28 (clone 37.51, 2.5 μg/mL)

-culture cells in supplemented complete RPMI 1640 (contains 10% FBS, 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, 50 μM β-mercaptoethanol)

- culture cells +/- recombinant human TGF-β1 and IL-2

3. Stimulate cells for 5 days

Week 2 (Day 5)

I. Responder cells

1. Isolate total mouse splenocytes from 2 mice using Splenocyte Purification procedure

2. Isolate total T cells from splenocytes using StemSep Mouse T Cell Enrichment Kit

3. Stain appropriate number of T cells with 1 μM CFSE using CFSE staining procedure

4. Check CFSE staining by flow

5. Stain for CD3 purity by flow

II. Potential Suppressor (Pooled Stimulated cells)

1. Pipet the appropriate cells from the plate on which they have been stimulated

2. Remove dead cells/debris using Lympholyte-M spin (see Protocol for Removal of Dead Cells using Lympholyte-M)

3. Perform cell counts with hemacytometer

III. Suppression Assay

1. The day before the Suppression Assay, Plate anti-mouse CD3+CD28 antibodies in 96-well flat-bottom plate [anti-CD3 (clone 500A2) at 0.5 μg/mL and anti-CD28 (clone 37.51) at 2.5 μg/mL in sterile PBS], incubate at 4°C overnight

2. Wash plate 3x with PBS

3. Add unlabeled potential Suppressor cells to the CFSE-labeled Responder cells at the proper ratios (1:2 Suppressor:Responder, with 300,000 cells per well total)

4. Plate cells for 3-5 days in 37°C incubator

5. On Day 3 or 5, wash cells once in staining buffer, resuspend in 200-400 μL staining buffer

6. Perform flow cytometry

7. Determine the % Proliferation of CFSE-labeled Responder cells

Protocol 11: Intracellular Cytokines Assay using Splenocytes from Mice Infected with VSV

-For acute response, spleens are taken from infected mice at day 7 post-infection

-For memory response, spleens are taken from infected mice at day 70 post-infection

1. Remove spleens from infected mice and one uninfected control (C57Bl/6)

    -See procedure for Purification of Mouse Splenocytes

2. Count cells in each sample with hemacytometer

3. Centrifuge samples (2 x 10^6 cells/sample, each mouse has 2 samples) at 2000 rpm, 3 minutes

4. Prepare stimulation media (while cells are spinning)

-Need (n x 400 µL) stimulation media with and without peptide (+ extra volume), n = # mice)

    -complete RPMI 1640 + 3 µM monensin (Golgi-stop) + 50 U/mL rIL-2 with or without 2 µg/mL MHC Class I specific VSV peptide

        -Add 1 µL monensin per mL

        -Add 5 µL rIL-2 per mL (rIL-2 stock is at 10U/µL)

        -To peptide medium: Add 1 µL peptide per mL (peptide stored at 2 µg/µL)

-Have n samples with peptide, n samples without peptide

5. Remove old media with a pipet

6. Resuspend each sample in 400 µL of appropriate medium (now at 5 x 10^6 cells/mL)

7. Plate samples (with and without peptide) in duplicate in 96-well round-bottom plates, 200 µL per well (1x 10^6 cells per well in duplicate wells, with and without peptide = 4 wells total per mouse)

8. Duplicate samples will be pooled for staining

9. Stimulate in 37°C incubator 5-6 hours

After 5-6 incubation:

10. Prepare Staining Buffer (DPBS + 0.5% BSA)

11. Prepare Blocking Solution [staining buffer + monensin + Fc receptor blocking Ab (anti-mouse CD16/32)]

-Pipet [(2n + 1) x 100 µL] staining buffer into an Eppendorf tube

-Add 1 µL/mL monensin

-Add 10 µL/mL blocking Ab

12. Pool duplicate samples from plate and centrifuge

13. Resuspend in 100 µL of Blocking Solution

14. Centrifuge 4 staining control samples from extra splenocytes (2 x 10^6 cells/sample)

-Resuspend these in 100 µL of plain staining buffer

-Add 1 µL blocking Ab per sample

15. Incubate 15 minutes on ice, centrifuge

16. Prepare Ab Solution (staining buffer + monensin + Abs)

-Pipet [(2n + 1) x 100 µL] staining buffer into an Eppendorf tube

-Add 1 µL/mL monensin

-Add 10 µL/mL of each Ab

-F4/80-PE

-CD8-PerCP

17. Resuspend stimulated samples in 100 µL of Ab Solution

18. Resuspend staining control samples in 100 µL of plain staining buffer

-Add 1 µL of appropriate Ab

-Single Staining controls:

-unstained

- ___-FITC (*e.g.* Thy1.2-FITC)

-F4/80-PE

-CD8-PerCP

19. Incubate 20 minutes, on ice, in dark

20. Centrifuge, resuspend samples in 100 µL Cytofix/Cytoperm Solution (BD Biosciences)

-(Resuspend single staining controls in 400 µL Staining Buffer + 4% paraformaldehyde. Now are finished with these controls.)

21. Incubate 20 minutes, on ice, in dark

22. Prepare 1x Perm/Wash Solution (BD Biosciences) by diluting 10x Perm/Wash solution in ddH_2O

23. Add 600 µL of 1x Perm/Wash Solution to each sample

24. Spin 2,000 rpm, 3 minutes

25. Use aspirator to remove all but ~100 µL of supernate, and use pipet to carefully remove the rest of the supernate

26. Prepare an anti-IFN-γ Solution

-Pipet [(2n + 1) x 100 µL] staining buffer into Eppendorf tube

-Add 10 µL/mL IFN-γ-FITC Ab

27. Resuspend cells in 100 µL of the IFN-γ Solution

28. Incubate 20 minutes, on ice, in dark

29. Centrifuge 2000 rpm, 3 minutes

30. Remove supernate with pipet, resuspend in 400 µL staining buffer + 4% paraformaldehyde

31. Analyze the cells the next morning by flow cytometry

Protocol 12: *In Vivo* Cytotoxicity Assay to Test Immune Response to VSV

1. Remove spleens from 2 TCRβ KO mice (donor mice)

2. Determine cell count using hemacytometer

3. Centrifuge volume containing 10 x 10^6 cells per recipient mouse

4. Resuspend cells in 1 mL diluent C (Sigma PKH26 dye kit)

5. Make 10 μM PKH solution in diluent C (stock PKH is 1 mM)

    -(990 μL diluent C + 10 μL PKH)

6. Add the 10 μM solution to the cell/diluent C solution, mix (5 μM final)

7. Incubate 5 minutes, room temperature

8. Add 1 mL FBS, incubate 1 minute

9. Add 1x volume (3 mL) complete RPMI 1640 1640

10. Spin slowly (40 speed on clinical centrifuge)

11. Resuspend in 10 mL complete RPMI 1640 1640

12. Split cell solution between 2 tubes (5 mL) each

13. Add _x_ mL complete RPMI 1640 to make concentration 2 x 10^6 cells/mL

14. Make CFSE dilutions for staining (CFSE stock = 5 mM)

    -Dilute stock 1:10 with DMSO (20 μL stock CFSE + 180 μL DMSO) = 500 μM

        -Take an aliquot and dilute it 1:10 (20 μL 500 μM CFSE + 180 μL DMSO)

            = 50 μM CFSE

    -Add 150 μL of the 500 μM CFSE solution to one tube (15 mL) of cell solution

        = 5 μM CFSE final concentration ($CFSE^{hi}$ group)

    -Add 150 μL of the 50 μM CFSE solution to second tube (15 mL) of cell solution

=0.5 μM CFSE final concentration ($CFSE^{lo}$ group)

15. Incubate 10 minutes in 37°C water bath

16. Centrifuge (5 minutes) and resuspend in 6 mL complete RPMI 1640 for each CFSE dilution group

17. Label 5 μM $CFSE^{hi}$ cell solution with 5 μg/mL peptide

18. Incubate both CFSE groups for 1 hour in 37°C incubator

19. Count cells from each group, centrifuge equal cell numbers for both groups

20. Resuspend in _x_ mL sterile PBS [x = (200 μL x # of recipient mice) + extra volume]

21. Pipet 200 μL from each CFSE group into __n_ Eppendorf tubes (n = # of recipient mice)

22. Inject 400 μL of the combined cell solution in PBS into infected mice and uninfected control mice

23. Harvest spleens 16 hours post-injection, centrifuge cells, resuspend in Staining Buffer + 4% paraformaldehyde

24. Perform flow cytometry